INDUCIBLE PROMOTERS

This application is a continuation-in-part of pending U.S. Application No. 10/760,752, filed January 20, 2004, which is a divisional of U.S. Application No. 09/733,685, filed December 8, 2000, which claims the benefit of U.S. Provisional Application No. 60/171,008, filed December 15, 1999, and U.S. Provisional Application No. 60/175,519, filed January 11, 2000, all of which are incorporated herein by reference in their entireties.

FIELD OF THE INVENTION

The present invention relates to broad-spectrum disease resistance in plants, including the phenomenon of systemic acquired resistance (SAR). More particularly, the present invention relates to the identification, isolation and characterization of genes encoding proteins that are involved in the regulation of SAR gene expression in plants.

BACKGROUND OF THE INVENTION

Plants are constantly challenged by a wide variety of pathogenic organisms including viruses, bacteria, fungi, and nematodes. Crop plants are particularly vulnerable because they are usually grown as genetically-uniform monocultures; when disease strikes, losses can be severe. However, most plants have their own innate mechanisms of defense against pathogenic organisms. Natural variation for resistance to plant pathogens has been identified by plant breeders and pathologists and bred into many crop plants. These natural disease resistance genes often provide high levels of resistance to or immunity against pathogens.

Systemic acquired resistance (SAR) is one component of the complex system plants use to defend themselves from pathogens (Hunt and Ryals, 1996; Ryals *et al.*, 1996). *See also,* U.S. Patent No. 5,614,395. SAR is a particularly important aspect of plant-pathogen responses because it is a pathogen-inducible, systemic resistance against a broad spectrum of infectious agents, including viruses, bacteria, and fungi. When the SAR signal transduction pathway is blocked, plants become more susceptible to pathogens that normally cause disease, and they also become susceptible to some infectious agents that would not normally cause

disease (Gaffney *et al.*, 1993; Delaney *et al.*, 1994; Delaney *et al.*, 1995; Delaney, 1997; Bi *et al.*, 1995; Mauch-Mani and Slusarenko, 1996). These observations indicate that the SAR signal transduction pathway is critical for maintaining plant health.

Conceptually, the SAR response can be divided into two phases. In the initiation phase, a pathogen infection is recognized, and a signal is released that travels through the phloem to distant tissues. This systemic signal is perceived by target cells, which react by expression of both SAR genes and disease resistance. The maintenance phase of SAR refers to the period of time, from weeks up to the entire life of the plant, during which the plant is in a quasi steady state, and disease resistance is maintained (Ryals *et al.*, 1996).

Salicylic acid (SA) accumulation appears to be required for SAR signal transduction. Plants that cannot accumulate SA due to treatment with specific inhibitors, epigenetic repression of phenylalanine ammonia-lyase, or transgenic expression of salicylate hydroxylase, which specifically degrades SA, also cannot induce either SAR gene expression or disease resistance (Gaffney *et al.*, 1993; Delaney *et al.*, 1994; Mauch-Mani and Slusarenko, 1996; Maher *et al.*, 1994; Pallas *et al.*, 1996). Although it has been suggested that SA might serve as the systemic signal, this is currently controversial and, to date, all that is known for certain is that if SA cannot accumulate, then SAR signal transduction is blocked (Pallas *et al.*, 1996; Shulaev *et al.*, 1995; Vernooij *et al.*, 1994).

Recently, *Arabidopsis* has emerged as a model system to study SAR (Uknes *et al.*, 1992; Uknes *et al.*, 1993; Cameron *et al.*, 1994; Mauch-Mani and Slusarenko, 1994; Dempsey and Klessig, 1995). It has been demonstrated that SAR can be activated in *Arabidopsis* by both pathogens and chemicals, such as SA, 2,6-dichloroisonicotinic acid (INA) and benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester (BTH) (Uknes *et al.*, 1992; Vernooij *et al.*, 1995; Lawton *et al.*, 1996). Following treatment with either INA or BTH or pathogen infection, at least three pathogenesis-related (PR) protein genes, namely, PR-1, PR-2, and PR-5 are coordinately induced concomitant with the onset of resistance (Uknes *et al.*, 1992, 1993). In tobacco, the best characterized species, treatment with a pathogen or an immunization compound induces the expression of at least nine sets of genes (Ward *et al.*, 1991). Transgenic disease-resistant plants have been created by transforming plants with various SAR genes (U.S. Patent No. 5,614,395).

A number of *Arabidopsis* mutants have been isolated that have modified SAR signal transduction (Delaney, 1997) The first of these mutants are the so-called *lsd* (lesions simulating disease) mutants and *acd2* (accelerated cell death) (Dietrich *et al.*, 1994; Greenberg *et al.*, 1994). These mutants all have some degree of spontaneous necrotic lesion formation on their leaves, elevated levels of SA, mRNA accumulation for the SAR genes, and significantly enhanced disease resistance. At least seven different *lsd* mutants have been isolated and characterized (Dietrich *et al.*, 1994; Weymann *et al.*, 1995). Another interesting class of mutants are *cim* (constitutive immunity) mutants (Lawton *et al.*, 1993). *See also,* U.S. Patent No. 5,792,904 and International PCT Application WO 94/16077. Like *lsd* mutants and *acd2*, *cim* mutants have elevated SA and SAR gene expression and resistance, but in contrast to *lsd* or *acd2,* do not display detectable lesions on their leaves. *cpr1* (constitutive expresser of PR genes) may be a type of *cim* mutant; however, because the presence of microscopic lesions on the leaves of *cpr1* has not been ruled out, *cpr1* might be a type of *lsd* mutant (Bowling *et al.*, 1994).

Mutants have also been isolated that are blocked in SAR signaling. *ndr1* (non-race-specific disease resistance) is a mutant that allows growth of both *Pseudomonas syringae* containing various avirulence genes and also normally avirulent isolates of *Peronospora parasitica* (Century *et al.*, 1995). Apparently this mutant is blocked early in SAR signaling. *npr1* (nonexpresser of PR genes) is a mutant that cannot induce expression of the SAR signaling pathway following INA treatment (Cao *et al.*, 1994). *eds* (enhanced disease susceptibility) mutants have been isolated based on their ability to support bacterial infection following inoculation of a low bacterial concentration (Glazebrook *et al.*, 1996; Parker *et al.*, 1996). Certain *eds* mutants are phenotypically very similar to *npr1,* and, recently, *eds5* and *eds53* have been shown to be allelic to *npr1* (Glazebrook *et al.*, 1996). *nim1* (noninducible immunity) is a mutant that supports *P. parasitica* (i.e., causal agent of downy mildew disease) growth following INA treatment (Delaney *et al.*, 1995; U.S. Patent No. 5,792,904). Although *nim1* can accumulate SA following pathogen infection, it cannot induce SAR gene expression or disease resistance, suggesting that the mutation blocks the pathway downstream of SA. *nim1* is also impaired in its ability to respond to INA or BTH, suggesting that the block exists downstream of the action of these chemicals (Delaney *et al.*, 1995; Lawton *et al.*, 1996).

Allelic *Arabidopsis* genes have been isolated and characterized, mutants of which are responsible for the *nim1* and *npr1* phenotypes, respectively (Ryals *et al.*, 1997; Cao *et al.*, 1997). The wild-type *NIM1* gene product is involved in the signal transduction cascade leading to both SAR and gene-for-gene disease resistance in *Arabidopsis* (Ryals *et al.*, 1997). Ryals *et al.*, 1997 also report the isolation of five additional alleles of *nim1* that show a range of phenotypes from weakly impaired in chemically induced PR-1 gene expression and fungal resistance to very strongly blocked. Transformation of the wild-type *NPR1* gene into *npr1* mutants not only complemented the mutations, restoring the responsiveness of SAR induction with respect to PR-gene expression and disease resistance, but also rendered the transgenic plants more resistant to infection by *P. syringae* in the absence of SAR induction (Cao *et al.*, 1997). WO 98/06748 describes the isolation of *NPR1* from *Arabidopsis* and a homologue from *Nicotiana glutinosa*. *See also,* WO 97/49822, WO 98/26082, and WO 98/29537.

Despite much research and the use of sophisticated and intensive crop protection measures, including genetic transformation of plants, losses due to disease remain in the billions of dollars annually. Therefore, there is a continuing need to develop new crop protection measures based on the ever-increasing understanding of the genetic basis for disease resistance in plants. In particular, there is a long-felt need for the identification, isolation, and characterization of more genes involved in the signal transduction cascade leading to systemic acquired resistance in plants.

SUMMARY OF THE INVENTION

The present invention addresses the aforementioned long-felt needs by using the *Arabidopsis NIM1* gene as bait in a two-hybrid screen to identify plant proteins that interact with the NIM1 protein. The novel NI16 and NI19 genes are obtainable by this screen. Homologues of the NI16 gene are obtainable by PCR amplification of total cellular DNA from other plants such as potato and tomato. The NI16 gene is rapidly induced following treatment with salicylic acid (SA) and benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester (BTH), as well as by pathogen inoculation. The NI16 gene is also induced in NIM1-overexpressing plants treated with BTH. Transgenic expression of the NI16 gene results in elevated levels of the pathogenesis-related (PR) protein PR-1. The amount of PR-1 induced

correlates with the amount of NI16 present, i.e., transgenic plants with the highest expression of NI16 have the highest levels of PR-1. It is believed that NI16 is a necessary component of the SAR response, and that overexpression of NI16 activates a subset of the response. PR-1 is also elevated in nahG and *nim1*-1 plants overexpressing NI16, which is significant because both *nim1* and nahG plants are normally deficient in their ability to induce PR-1 in response to pathogens. Thus, the NI16 gene encodes a protein involved in the regulation of SAR gene expression in plants. The NI16 gene can be expressed in transgenic plants, either alone or in combination with the NIM1 protein, to enhance expression of SAR genes such as PR-1.

In particular, the present invention is directed to an isolated nucleic acid molecule comprising a sequence that encodes the amino acid sequence of SEQ ID NO:2, SEQ ID NO:5, SEQ ID NO:7, or SEQ ID NO:23.

The present invention is further directed to an isolated nucleic acid molecule comprising the coding region of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:6, or SEQ ID NO:22.

The present invention is also directed to an isolated nucleic acid molecule comprising a sequence that encodes a protein involved in the signal transduction cascade leading to systemic acquired resistance in plants, wherein said sequence comprises a 20, 25, 30, 35, 40, 45, or 50 (preferably 20) base pair nucleotide portion identical in sequence to a respective consecutive 20, 25, 30, 35, 40, 45, or 50 (preferably 20) base pair nucleotide portion of the coding region of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:6, or SEQ ID NO:22.

The present invention is further directed to an isolated nucleic acid molecule comprising a nucleotide sequence that encodes a protein involved in the signal transduction cascade leading to systemic acquired resistance in plants, wherein said nucleotide sequence can be amplified from total cellular DNA from a plant using the polymerase chain reaction with the primers shown as SEQ ID NO:20 and SEQ ID NO:21.

The present invention also encompasses a chimeric gene comprising a promoter active in plants operatively linked to a coding sequence of the present invention, a recombinant vector comprising such a chimeric gene, wherein the vector is capable of being stably transformed into a host, as well as a host stably transformed with such a vector. Preferably, the host is a plant such as one of the following agronomically important crops: rice, wheat, barley, rye, rape, corn, potato, carrot, sweet potato, sugar beet, bean, pea, chicory, lettuce,

cabbage, cauliflower, broccoli, turnip, radish, spinach, asparagus, onion, garlic, eggplant, pepper, celery, squash, pumpkin, cucumber, apple, pear, quince, melon, plum, cherry, peach, nectarine, apricot, strawberry, grape, raspberry, blackberry, pineapple, avocado, papaya, mango, banana, soybean, tobacco, tomato, sorghum, and sugarcane.

In another aspect, the present invention provides the nucleic acid sequence of an NI16 promoter. In one embodiment, the NI16 promoter comprises SEQ ID NO:24 (962 bp). In another embodiment, the NI16 promoter comprises a fragment of SEQ ID NO:24. Exemplary fragments of SEQ ID NO:24 include, but are not limited to, the region upstream of nucleotide number 863 of SEQ ID NO:3 (i.e., SEQ ID NO:25), SEQ ID NO:26 (274 bp), and SEQ ID NO:27 (544 bp). In another embodiment, the NI16 promoter comprises SEQ ID NO:28 (274 bp).

In addition, the present invention encompasses chimeric genes comprising the NI16 promoter, or a fragment thereof, operatively linked to a coding sequence of a gene of interest, wherein the NI16 promoter or fragment thereof regulates transcription of the coding sequence in the presence of chemical regulators. In a preferred embodiment, the coding sequence encodes an enzyme, such as an assayable marker, whereby expression of the marker can be observed in assays for chemical induction of the chimeric gene. In related aspects, the present invention also embodies a recombinant vector, such as a plasmid, comprising the aforementioned chimeric gene, as well as a plant or plant tissue stably transformed with such a chimeric gene.

BRIEF DESCRIPTION OF THE SEQUENCES IN THE SEQUENCE LISTING

SEQ ID NO:1 - Full length cDNA sequence of the *Arabidopsis thaliana* NI16 gene.

SEQ ID NO:2 - Amino acid sequence of the NI16 protein encoded by SEQ ID NO:1.

SEQ ID NO:3 - Genomic DNA sequence of the *Arabidopsis thaliana* NI16 gene region, including the 5' upstream promoter sequence.

SEQ ID NO:4 - Full length cDNA sequence of the potato NI16 homologue.

SEQ ID NO:5 - Potato NI16 amino acid sequence encoded by SEQ ID NO:4.

SEQ ID NO:6 - Partial cDNA sequence of the tomato NI16 homologue.

SEQ ID NO:7 - Tomato NI16 amino acid sequence encoded by SEQ ID NO:6.

SEQ ID NO:8 - Amino acid sequence encoded by soybean EST AI495102.

SEQ ID NO:9 - Amino acid sequence encoded by soybean EST AI461039.

SEQ ID NO:10 - Amino acid sequence encoded by tobacco G8-1 gene.

SEQ ID NO:11 - Primer *NIM*5'RI

SEQ ID NO:12 - Primer *NIM*3'SalI

SEQ ID NO:13 - Primer *NIM*trunc3'NcoI

SEQ ID NO:14 - Primer *NIM*loop5'RI

SEQ ID NO:15 - Primer 16GSP1

SEQ ID NO:16 - Primer 16GSP2

SEQ ID NO:17 - Primer 16GSP3

SEQ ID NO:18 - Primer 16F

SEQ ID NO:19 - Primer 16R

SEQ ID NO:20 - Primer NI16-DegF

SEQ ID NO:21 - Primer NI16-DegR

SEQ ID NO:22 - Genomic sequence of the *Arabidopsis thaliana* NI19 gene.

SEQ ID NO:23 - Amino acid sequence of the NI19 protein encoded by SEQ ID NO:22.

SEQ ID NO:24 – NI16 (962 bp) promoter sequence

SEQ ID NO:25 – NI16 (862 bp) promoter sequence

SEQ ID NO:26 – NI16 (274 bp) promoter sequence

SEQ ID NO:27 - NI16 (544 bp) promoter sequence

SEQ ID NO:28 – NI16 (274 bp) promoter sequence

SEQ ID NO:29 – DNA sequence encoding GUS reporter with an intron sequence (i.e., GIG)

SEQ ID NO:30 - AtPR1 promoter

SEQ ID NO:31 – SMAS promoter

DEFINITIONS

In describing the present invention, the following terms will be employed, and are intended to be defined as indicated below.

Associated With / Operatively Linked: Refers to two DNA sequences that are related physically or functionally. For example, a promoter or regulatory DNA sequence is said to be

"associated with" a DNA sequence that codes for an RNA or a protein if the two sequences are operatively linked, or situated such that the regulator DNA sequence will affect the expression level of the coding or structural DNA sequence.

Chimeric Gene: A recombinant DNA sequence in which a promoter or regulatory DNA sequence is operatively linked to, or associated with, a DNA sequence that codes for an mRNA or which is expressed as a protein, such that the regulator DNA sequence is able to regulate transcription or expression of the associated DNA sequence. The regulator DNA sequence of the chimeric gene is not normally operatively linked to the associated DNA sequence as found in nature.

Coding Sequence: a nucleic acid sequence that is transcribed into RNA such as mRNA, rRNA, tRNA, snRNA, sense RNA or antisense RNA. Preferably the RNA is then translated in an organism to produce a protein.

Complementary: refers to two nucleotide sequences that comprise antiparallel nucleotide sequences capable of pairing with one another upon formation of hydrogen bonds between the complementary base residues in the antiparallel nucleotide sequences.

Expression: refers to the transcription and/or translation of an endogenous gene or a transgene in plants. In the case of antisense constructs, for example, expression may refer to the transcription of the antisense DNA only.

Expression Cassette: A nucleic acid sequence capable of directing expression of a particular nucleotide sequence in an appropriate host cell, comprising a promoter operably linked to the nucleotide sequence of interest which is operably linked to termination signals. It also typically comprises sequences required for proper translation of the nucleotide sequence. The expression cassette comprising the nucleotide sequence of interest may be chimeric, meaning that at least one of its components is heterologous with respect to at least one of its other components. The expression cassette may also be one which is naturally occurring but has been obtained in a recombinant form useful for heterologous expression. Typically, however, the expression cassette is heterologous with respect to the host, i.e., the particular nucleic acid sequence of the expression cassette does not occur naturally in the host cell and must have been introduced into the host cell or an ancestor of the host cell by a transformation event. The expression of the nucleotide sequence in the expression cassette may be under the control of a constitutive promoter or of an inducible promoter which initiates transcription

only when the host cell is exposed to some particular external stimulus. In the case of a multicellular organism, such as a plant, the promoter can also be specific to a particular tissue, or organ, or stage of development.

Gene: A defined region that is located within a genome and that, besides the aforementioned coding nucleic acid sequence, comprises other, primarily regulatory, nucleic acid sequences responsible for the control of expression, i.e., transcription and translation of the coding portion. A gene may also comprise other 5' and 3' untranslated sequences and termination sequences. Further elements that may be present are, for example, introns.

Heterologous DNA Sequence: The terms "heterologous DNA sequence", "exogenous DNA segment" or "heterologous nucleic acid," as used herein, each refer to a sequence that originates from a source foreign to the particular host cell or, if from the same source, is modified from its original form. Thus, a heterologous gene in a host cell includes a gene that is endogenous to the particular host cell but has been modified through, for example, the use of DNA shuffling. The terms also includes non-naturally occurring multiple copies of a naturally occurring DNA sequence. Thus, the terms refer to a DNA segment that is foreign or heterologous to the cell, or homologous to the cell but in a position within the host cell nucleic acid in which the element is not ordinarily found. Exogenous DNA segments are expressed to yield exogenous polypeptides.

Homologous DNA Sequence: A DNA sequence naturally associated with a host cell into which it is introduced.

Isocoding: A nucleic acid sequence is isocoding with a reference nucleic acid sequence when the nucleic acid sequence encodes a polypeptide having the same amino acid sequence as the polypeptide encoded by the reference nucleic acid sequence.

Isolated: In the context of the present invention, an isolated nucleic acid molecule or an isolated enzyme is a nucleic acid molecule or enzyme that, by the hand of man, exists apart from its native environment and is therefore not a product of nature. An isolated nucleic acid molecule or enzyme may exist in a purified form or may exist in a non-native environment such as, for example, a recombinant host cell.

Minimal Promoter: a promoter element, particularly a TATA element, that is inactive or has greatly reduced promoter activity in the absence of upstream activation. In the presence of a suitable transcription factor, a minimal promoter functions to permit transcription.

Native: refers to a gene that is present in the genome of an untransformed cell.

Naturally occurring: the term "naturally occurring" is used to describe an object that can be found in nature as distinct from being artificially produced by man. For example, a protein or nucleotide sequence present in an organism (including a virus), which can be isolated from a source in nature and which has not been intentionally modified by man in the laboratory, is naturally occurring.

NI16: Gene involved in the SAR signal transduction cascade that interacts with the NIM1 protein.

NIM1: Gene described in Ryals *et al.*, 1997, which is involved in the SAR signal transduction cascade.

NIM1: Protein encoded by the *NIM1* gene.

Nucleic acid: the term "nucleic acid" refers to deoxyribonucleotides or ribonucleotides and polymers thereof in either single- or double-stranded form. Unless specifically limited, the term encompasses nucleic acids containing known analogues of natural nucleotides which have similar binding properties as the reference nucleic acid and are metabolized in a manner similar to naturally occurring nucleotides. Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof *(e.g.* degenerate codon substitutions) and complementary sequences and as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer *et al., Nucleic Acid Res.* 19: 5081 (1991); Ohtsuka *et al., J. Biol. Chem.* 260: 2605-2608 (1985); Rossolini *et al., Mol. Cell. Probes* 8: 91-98 (1994)). The terms "nucleic acid" or "nucleic acid sequence" may also be used interchangeably with gene, cDNA, and mRNA encoded by a gene. In the context of the present invention, the nucleic acid molecule is preferably a segment of DNA. Nucleotides are indicated by their bases by the following standard abbreviations: adenine (A), cytosine (C), thymine (T), and guanine (G).

ORF: Open Reading Frame.

Plant: Any whole plant.

Plant Cell: Structural and physiological unit of a plant, comprising a protoplast and a cell wall. The plant cell may be in form of an isolated single cell or a cultured cell, or as a part of higher organized unit such as, for example, a plant tissue, a plant organ, or a whole plant.

Plant Cell Culture: Cultures of plant units such as, for example, protoplasts, cell culture cells, cells in plant tissues, pollen, pollen tubes, ovules, embryo sacs, zygotes and embryos at various stages of development.

Plant Material: Refers to leaves, stems, roots, flowers or flower parts, fruits, pollen, egg cells, zygotes, seeds, cuttings, cell or tissue cultures, or any other part or product of a plant.

Plant Organ: A distinct and visibly structured and differentiated part of a plant such as a root, stem, leaf, flower bud, or embryo.

Plant tissue: A group of plant cells organized into a structural and functional unit. Any tissue of a plant *in planta* or in culture is included. This term includes, but is not limited to, whole plants, plant organs, plant seeds, tissue culture and any groups of plant cells organized into structural and/or functional units. The use of this term in conjunction with, or in the absence of, any specific type of plant tissue as listed above or otherwise embraced by this definition is not intended to be exclusive of any other type of plant tissue.

Promoter: An untranslated DNA sequence upstream of the coding region that contains the binding site for RNA polymerase II and initiates transcription of the DNA. The promoter region may also include other elements that act as regulators of gene expression.

Protoplast: An isolated plant cell without a cell wall or with only parts of the cell wall.

Purified: the term "purified," when applied to a nucleic acid or protein, denotes that the nucleic acid or protein is essentially free of other cellular components with which it is associated in the natural state. It is preferably in a homogeneous state although it can be in either a dry or aqueous solution. Purity and homogeneity are typically determined using analytical chemistry techniques such as polyacrylamide gel electrophoresis or high performance liquid chromatography. A protein which is the predominant species present in a preparation is substantially purified. The term "purified" denotes that a nucleic acid or protein gives rise to essentially one band in an electrophoretic gel. Particularly, it means that the nucleic acid or protein is at least about 50% pure, more preferably at least about 85% pure, and most preferably at least about 99% pure.

Recombinant DNA molecule: a combination of DNA molecules that are joined together using recombinant DNA technology

Regulatory Elements: Sequences involved in controlling the expression of a nucleotide sequence. Regulatory elements comprise a promoter operably linked to the nucleotide sequence of interest and termination signals. They also typically encompass sequences required for proper translation of the nucleotide sequence.

Selectable marker gene: a gene whose expression in a plant cell gives the cell a selective advantage. The selective advantage possessed by the cells transformed with the selectable marker gene may be due to their ability to grow in the presence of a negative selective agent, such as an antibiotic or a herbicide, compared to the growth of non-transformed cells. The selective advantage possessed by the transformed cells, compared to non-transformed cells, may also be due to their enhanced or novel capacity to utilize an added compound as a nutrient, growth factor or energy source. Selectable marker gene also refers to a gene or a combination of genes whose expression in a plant cell gives the cell both, a negative and a positive selective advantage.

Significant Increase: an increase in enzymatic activity that is larger than the margin of error inherent in the measurement technique, preferably an increase by about 2-fold or greater of the activity of the wild-type enzyme in the presence of the inhibitor, more preferably an increase by about 5-fold or greater, and most preferably an increase by about 10-fold or greater.

The terms "identical" or percent "identity" in the context of two or more nucleic acid or protein sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues or nucleotides that are the same, when compared and aligned for maximum correspondence, as measured using one of the following sequence comparison algorithms or by visual inspection.

Substantially identical: the phrase "substantially identical," in the context of two nucleic acid or protein sequences, refers to two or more sequences or subsequences that have at least 60%, preferably 80%, more preferably 90-95%, and most preferably at least 99% nucleotide or amino acid residue identity, when compared and aligned for maximum correspondence, as measured using one of the following sequence comparison algorithms or by visual inspection. Preferably, the substantial identity exists over a region of the sequences that is at least about

50 residues in length, more preferably over a region of at least about 100 residues, and most preferably the sequences are substantially identical over at least about 150 residues. In a most preferred embodiment, the sequences are substantially identical over the entire length of the coding regions. Furthermore, substantially identical nucleic acid or protein sequences perform substantially the same function.

For sequence comparison, typically one sequence acts as a reference sequence to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are input into a computer, subsequence coordinates are designated if necessary, and sequence algorithm program parameters are designated. The sequence comparison algorithm then calculates the percent sequence identity for the test sequence(s) relative to the reference sequence, based on the designated program parameters.

Optimal alignment of sequences for comparison can be conducted, e.g., by the local homology algorithm of Smith & Waterman, *Adv. Appl. Math.* 2: 482 (1981), by the homology alignment algorithm of Needleman & Wunsch, *J. Mol. Biol.* 48: 443 (1970), by the search for similarity method of Pearson & Lipman, *Proc. Nat'l. Acad. Sci. USA* 85: 2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by visual inspection (*see generally,* Ausubel *et al., infra).*

One example of an algorithm that is suitable for determining percent sequence identity and sequence similarity is the BLAST algorithm, which is described in Altschul *et al., J. Mol. Biol.* 215: 403-410 (1990). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul *et al.*, 1990). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are then extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always > 0) and N (penalty score for mismatching residues; always < 0). For amino

acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when the cumulative alignment score falls off by the quantity X from its maximum achieved value, the cumulative score goes to zero or below due to the accumulation of one or more negative-scoring residue alignments, or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) of 10, a cutoff of 100, M=5, N=-4, and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength (W) of 3, an expectation (E) of 10, and the BLOSUM62 scoring matrix *(see* Henikoff & Henikoff, *Proc. Natl. Acad. Sci. USA* 89: 10915 (1989)).

In addition to calculating percent sequence identity, the BLAST algorithm also performs a statistical analysis of the similarity between two sequences *(see, e.g.,* Karlin & Altschul, *Proc. Nat'l. Acad. Sci. USA* 90: 5873-5787 (1993)). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a test nucleic acid sequence is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid sequence to the reference nucleic acid sequence is less than about 0.1, more preferably less than about 0.01, and most preferably less than about 0.001.

Another indication that two nucleic acid sequences are substantially identical is that the two molecules hybridize to each other under stringent conditions. The phrase "hybridizing specifically to" refers to the binding, duplexing, or hybridizing of a molecule only to a particular nucleotide sequence under stringent conditions when that sequence is present in a complex mixture (*e.g.,* total cellular) DNA or RNA. "Bind(s) substantially" refers to complementary hybridization between a probe nucleic acid and a target nucleic acid and embraces minor mismatches that can be accommodated by reducing the stringency of the hybridization media to achieve the desired detection of the target nucleic acid sequence.

"Stringent hybridization conditions" and "stringent hybridization wash conditions" in the context of nucleic acid hybridization experiments such as Southern and Northern hybridizations are sequence dependent, and are different under different environmental parameters. Longer sequences hybridize specifically at higher temperatures. An extensive

guide to the hybridization of nucleic acids is found in Tijssen (1993) *Laboratory Techniques in Biochemistry and Molecular Biology-Hybridization with Nucleic* Acid Probes part I chapter 2 "Overview of principles of hybridization and the strategy of nucleic acid probe assays" Elsevier, New York. Generally, highly stringent hybridization and wash conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. Typically, under "stringent conditions" a probe will hybridize to its target subsequence, but to no other sequences.

The T_m is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Very stringent conditions are selected to be equal to the T_m for a particular probe. An example of stringent hybridization conditions for hybridization of complementary nucleic acids which have more than 100 complementary residues on a filter in a Southern or northern blot is 50% formamide with 1 mg of heparin at 42°C, with the hybridization being carried out overnight. An example of highly stringent wash conditions is 0.1 5M NaCl at 72°C for about 15 minutes. An example of stringent wash conditions is a 0.2x SSC wash at 65°C for 15 minutes (*see,* Sambrook, *infra*, for a description of SSC buffer). Often, a high stringency wash is preceded by a low stringency wash to remove background probe signal. An example medium stringency wash for a duplex of, e.g., more than 100 nucleotides, is 1x SSC at 45°C for 15 minutes. An example low stringency wash for a duplex of, *e.g.,* more than 100 nucleotides, is 4-6x SSC at 40°C for 15 minutes. For short probes (*e.g.,* about 10 to 50 nucleotides), stringent conditions typically involve salt concentrations of less than about 1.0M Na ion, typically about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7.0 to 8.3, and the temperature is typically at least about 30°C. Stringent conditions can also be achieved with the addition of destabilizing agents such as formamide. In general, a signal to noise ratio of 2x (or higher) than that observed for an unrelated probe in the particular hybridization assay indicates detection of a specific hybridization. Nucleic acids that do not hybridize to each other under stringent conditions are still substantially identical if the proteins that they encode are substantially identical. This occurs, e.g., when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code.

The following are examples of sets of hybridization/wash conditions that may be used to clone homologous nucleotide sequences that are substantially identical to reference nucleotide

sequences of the present invention: a reference nucleotide sequence preferably hybridizes to the reference nucleotide sequence in 7% sodium dodecyl sulfate (SDS), 0.5 M $NaPO_4$, 1 mM EDTA at 50°C with washing in 2X SSC, 0.1% SDS at 50°C, more desirably in 7% sodium dodecyl sulfate (SDS), 0.5 M $NaPO_4$, 1 mM EDTA at 50°C with washing in 1X SSC, 0.1% SDS at 50°C, more desirably still in 7% sodium dodecyl sulfate (SDS), 0.5 M $NaPO_4$, 1 mM EDTA at 50°C with washing in 0.5X SSC, 0.1% SDS at 50°C, preferably in 7% sodium dodecyl sulfate (SDS), 0.5 M $NaPO_4$, 1 mM EDTA at 50°C with washing in 0.1X SSC, 0.1% SDS at 50°C, more preferably in 7% sodium dodecyl sulfate (SDS), 0.5 M $NaPO_4$, 1 mM EDTA at 50°C with washing in 0.1X SSC, 0.1% SDS at 65°C.

A further indication that two nucleic acid sequences or proteins are substantially identical is that the protein encoded by the first nucleic acid is immunologically cross reactive with, or specifically binds to, the protein encoded by the second nucleic acid. Thus, a protein is typically substantially identical to a second protein, for example, where the two proteins differ only by conservative substitutions.

The phrase "specifically (or selectively) binds to an antibody," or "specifically (or selectively) immunoreactive with," when referring to a protein or peptide, refers to a binding reaction which is determinative of the presence of the protein in the presence of a heterogeneous population of proteins and other biologics. Thus, under designated immunoassay conditions, the specified antibodies bind to a particular protein and do not bind in a significant amount to other proteins present in the sample. Specific binding to an antibody under such conditions may require an antibody that is selected for its specificity for a particular protein. For example, antibodies raised to the protein with the amino acid sequence encoded by any of the nucleic acid sequences of the invention can be selected to obtain antibodies specifically immunoreactive with that protein and not with other proteins except for polymorphic variants. A variety of immunoassay formats may be used to select antibodies specifically immunoreactive with a particular protein. For example, solid-phase ELISA immunoassays, Western blots, or immunohistochemistry are routinely used to select monoclonal antibodies specifically immunoreactive with a protein. See Harlow and Lane (1988) *Antibodies, A Laboratory Manual,* Cold Spring Harbor Publications, New York "Harlow and Lane"), for a description of immunoassay formats and conditions that can be used to determine specific immunoreactivity. Typically a specific or selective reaction will be

at least twice background signal or noise and more typically more than 10 to 100 times background.

"Conservatively modified variations" of a particular nucleic acid sequence refers to those nucleic acid sequences that encode identical or essentially identical amino acid sequences, or where the nucleic acid sequence does not encode an amino acid sequence, to essentially identical sequences. Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given polypeptide. For instance the codons CGT, CGC, CGA, CGG, AGA, and AGG all encode the amino acid arginine. Thus, at every position where an arginine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded protein. Such nucleic acid variations are "silent variations" which are one species of "conservatively modified variations." Every nucleic acid sequence described herein which encodes a protein also describes every possible silent variation, except where otherwise noted. One of skill will recognize that each codon in a nucleic acid (except ATG, which is ordinarily the only codon for methionine) can be modified to yield a functionally identical molecule by standard techniques. Accordingly, each "silent variation" of a nucleic acid which encodes a protein is implicit in each described sequence.

Furthermore, one of skill will recognize that individual substitutions deletions or additions that alter, add or delete a single amino acid or a small percentage of amino acids (typically less than 5%, more typically less than 1%) in an encoded sequence are "conservatively modified variations," where the alterations result in the substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art. The following five groups each contain amino acids that are conservative substitutions for one another: Aliphatic: Glycine (G), Alanine (A), Valine (V), Leucine (L), Isoleucine (I); Aromatic: Phenylalanine (F), Tyrosine (Y), Tryptophan (W); Sulfur-containing: Methionine (M), Cysteine (C); Basic: Arginine (R), Lysine (K), Histidine (H); Acidic: Aspartic acid (D), Glutamic acid (E), Asparagine (N), Glutamine (Q). *See also,* Creighton (1984) *Proteins, W.H.* Freeman and Company. In addition, individual substitutions, deletions or additions which alter, add or delete a single amino acid or a small percentage of amino acids in an encoded sequence are also "conservatively modified variations."

A "subsequence" refers to a sequence of nucleic acids or amino acids that comprise a part of a longer sequence of nucleic acids or amino acids (e.g., protein) respectively.

Nucleic acids are "elongated" when additional nucleotides (or other analogous molecules) are incorporated into the nucleic acid. Most commonly, this is performed with a polymerase (e.g., a DNA polymerase), e.g., a polymerase which adds sequences at the 3' terminus of the nucleic acid.

Two nucleic acids are "recombined" when sequences from each of the two nucleic acids are combined in a progeny nucleic acid. Two sequences are "directly" recombined when both of the nucleic acids are substrates for recombination. Two sequences are "indirectly recombined" when the sequences are recombined using an intermediate such as a cross-over oligonucleotide. For indirect recombination, no more than one of the sequences is an actual substrate for recombination, and in some cases, neither sequence is a substrate for recombination.

A "specific binding affinity" between two molecules, for example, a ligand and a receptor, means a preferential binding of one molecule for another in a mixture of molecules. The binding of the molecules can be considered specific if the binding affinity is about 1 x 10^4 M^{-1} to about 1 x 10^6 M^{-1} or greater.

Transformation: a process for introducing heterologous DNA into a host cell or organism.

"Transformed," "transgenic," and "recombinant" refer to a host organism such as a bacterium or a plant into which a heterologous nucleic acid molecule has been introduced. The nucleic acid molecule can be stably integrated into the genome of the host or the nucleic acid molecule can also be present as an extrachromosomal molecule. Such an extrachromosomal molecule can be auto-replicating. Transformed cells, tissues, or plants are understood to encompass not only the end product of a transformation process, but also transgenic progeny thereof. A "non-transformed," "non-transgenic," or "non-recombinant" host refers to a wild-type organism, e.g., a bacterium or plant, which does not contain the heterologous nucleic acid molecule.

DEPOSIT INFORMATION

The following material has been deposited with the Agricultural Research Service, Patent Culture Collection (NRRL), 1815 North University Street, Peoria, Illinois 61604, USA, under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure. All restrictions on the availability of the deposited material will be irrevocably removed upon the granting of a patent.

Clone	Accession Number	Date of Deposit
pRC25	NRRL B-30239	January 7, 2000

DETAILED DESCRIPTION OF THE INVENTION

The *Arabidopsis NIM1* gene (Ryals *et al.,* 1997) is used as a bait in a yeast two-hybrid screen to isolate the novel NIM1 interactors NI16 and NI19. The NI16 gene is rapidly induced following treatment with salicylic acid (SA) and benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester (BTH), as well as by pathogen inoculation. The NI16 gene is also induced in NIM1-overexpressing plants treated with BTH. The NI16 gene sequence contains regions of high homology to the tobacco G8-1 gene, which is induced rapidly following treatment with salicylic acid, and which is responsive to very low concentrations of salicylate.

A full length *Arabidopsis* NI16 cDNA is determined by 5' RACE kit and encodes a protein of 122 amino acids. In addition, a genomic copy of *Arabidopsis* NI16 is cloned from a genomic library. The genomic clone also contains the NI16 5' upstream promoter region, which includes several promoter elements in the region adjacent to the NI16 genomic coding region. These promoter elements include: a tandem CaMV AS1 motif, which is known to be SA inducible (Terzaghi and Cashmore, 1995); a tandem TCA1 motif, which is another common SA inducible element (Goldsbrough *et al.,* 1993); a MYCATR22 element, which is a binding site for MCY (rd22BPI) in *Arabidopsis* dehydration-responsive gene rd22 (ABA-induction); a PAL BOX, which is one of three putative cis-acting elements of the phenylalanine ammonia-lyase family in parsley; and a HEXAMERAT4 element, which is a hexamer motif of the *Arabidopsis* histone H4 promoter. Because the NI16 gene is rapidly induced following treatment with the SAR inducers SA and BTH, it is predicted that the NI16

promoter is inducible by a full range of known SAR-inducers and can therefore be used for the same purposes as described for the tobacco PR-1a promoter in U.S. Patent No. 5,614,395 and for the *Arabidopsis* PR-1 promoter in WO 98/03536.

NI16 and NI19 gene sequences according to the invention can be isolated using the techniques described in the examples below, or by PCR using the NI16 sequences (SEQ ID NO:1 or SEQ ID NO:3) or NI19 sequence (SEQ ID NO:22) set forth in the sequence listing as the basis for constructing PCR primers. For example, oligonucleotides corresponding to the sequence of approximately the first and last 20-25 consecutive nucleotides of the NI16 coding sequence or their complements (e.g., SEQ ID NO:20 and SEQ ID NO:21) can be used as PCR primers to amplify a NI16 gene sequence directly from a cDNA or genomic library from a source plant such as *Arabidopsis thaliana*, potato or tomato. Thus, the NI16 and NI19 gene sequences set forth in the sequence listing, as well as homologous NI16 and NI19 gene sequences from additional plants, can likewise be amplified by PCR from cDNA and genomic libraries.

Transgenic expression of the NI16 gene results in constitutive expression of PR-1. Hence, the NI16 gene encodes a protein involved in the regulation of SAR gene expression.

A NI16 or NI19 coding sequence of the present invention may be inserted into an expression cassette designed for plants to construct a chimeric gene according to the invention using standard genetic engineering techniques. The choice of specific regulatory sequences such as promoter, signal sequence, 5' and 3' untranslated sequences, and enhancer appropriate for the achieving the desired pattern and level of expression in the chosen plant host is within the level of skill of the routineer in the art. The resultant molecule, containing the individual elements linked in proper reading frame, may be inserted into a vector capable of being transformed into a host plant cell.

Examples of promoters capable of functioning in plants or plant cells (i.e., those capable of driving expression of associated coding sequences such as those coding for the NI16 or NI19 protein in plant cells) include the *Arabidopsis* and maize ubiquitin promoters; cauliflower mosaic virus (CaMV) 19S or 35S promoters and CaMV double promoters; rice actin promoters; PR-1 promoters from tobacco, *Arabidopsis*, or maize; nopaline synthase promoters; small subunit of ribulose bisphosphate carboxylase (ssuRUBISCO) promoters, and the like. Especially preferred is the *Arabidopsis* ubiquitin promoter. The promoters

themselves may be modified to manipulate promoter strength to increase expression of the associated coding sequence in accordance with art-recognized procedures. Preferred promoters for use with the present invention are those that confer high level constitutive expression.

Signal or transit peptides may be fused to the NI16 coding sequence in the chimeric DNA constructs of the invention to direct transport of the expressed protein to the desired site of action. Examples of signal peptides include those natively linked to the plant pathogenesis-related proteins, e.g. PR-1, PR-2, and the like. *See, e.g.,* Payne *et al.,* 1988. Examples of transit peptides include the chloroplast transit peptides such as those described in Von Heijne *et al.* (1991), *Mazur et al.* (1987), and Vorst *et al.* (1988); and mitochondrial transit peptides such as those described in Boutry *et al.* (1987). Also included are sequences that result in localization of the encoded protein to various cellular compartments such as the vacuole. See, for example, Neuhaus *et al.* (1991) and Chrispeels (1991).

The chimeric DNA construct(s) of the invention may contain multiple copies of a promoter or multiple copies of a NI16 or NI19 coding sequence of the present invention. In addition, the construct(s) may include coding sequences for markers and coding sequences for other peptides such as signal or transit peptides, each in proper reading frame with the other functional elements in the DNA molecule. The preparation of such constructs are within the ordinary level of skill in the art.

Useful markers include peptides providing herbicide, antibiotic or drug resistance, such as, for example, resistance to protoporphyrinogen oxidase inhibitors, hygromycin, kanamycin, G418, gentamycin, lincomycin, methotrexate, glyphosate, phosphinothricin, or the like. These markers can be used to select cells transformed with the chimeric DNA constructs of the invention from untransformed cells. Other useful markers are peptidic enzymes which can be easily detected by a visible reaction, for example a color reaction, for example luciferase, ß-glucuronidase, or ß-galactosidase.

Chimeric genes designed for plant expression such as those described herein can be introduced into the plant cell in a number of art-recognized ways. Those skilled in the art will appreciate that the choice of method might depend on the type of plant (i.e. monocot or dicot) and/or organelle (i.e. nucleus, chloroplast, mitochondria) targeted for transformation. Suitable methods of transforming plant cells include microinjection (Crossway *et al.,* 1986),

electroporation (Riggs *et al.,* 1986), *Agrobacterium* mediated transformation (Hinchee *et al.,* 1988; Ishida *et al.,* 1996), direct gene transfer (Paszkowski *et al.,* 1984; Hayashimoto *et al.,* 1990), and ballistic particle acceleration using devices available from Agracetus, Inc., Madison, Wisconsin and Dupont, Inc., Wilmington, Delaware (*see, for example,* U.S. Patent 4,945,050; and McCabe *et al.,* 1988). S*ee also*, Weissinger *et al.* (1988); Sanford *et al.* (1987) (onion); Christou *et al.* (1988) (soybean); McCabe *et al.* (1988) (soybean); Datta *et al.* (1990) (rice); Klein *et al.* (1988) (maize); Klein *et al.* (1988) (maize); Klein *et al.* (1988) (maize); Fromm *et al.* (1990); and Gordon-Kamm *et al.* (1990) (maize); Svab *et al.* (1990) (tobacco chloroplasts); Gordon-Kamm *et al.* (1993) (maize); Shimamoto *et al.* (1989) (rice); Christou *et al.* (1991) (rice); Datta *et al.* (1990) (rice); European Patent Application EP 0 332 581 (orchardgrass and other *Pooideae*); Vasil *et al.* (1993) (wheat); Weeks *et al.* (1993) (wheat); Wan *et al.* (1994) (barley); Jahne *et al.* (1994) (barley); Umbeck *et al.* (1987) (cotton); Casas *et al.* (1993) (sorghum); Somers *et al.* (1992) (oats); Torbert *et al.* (1995) (oats); Weeks *et al.*,(1993) (wheat); WO 94/13822 (wheat); and Nehra *et al.* (1994) (wheat). A particularly preferred set of embodiments for the introduction of recombinant DNA molecules into maize by microprojectile bombardment can be found in Koziel *et al.* (1993); Hill *et al.* (1995) and Koziel *et al.* (1996). An additional preferred embodiment is the protoplast transformation method for maize as disclosed in EP 0 292 435.

Once a chimeric gene comprising a NI16 or NI19 coding sequence has been transformed into a particular plant species, it may be propagated in that species or moved into other varieties of the same species, particularly including commercial varieties, using traditional breeding techniques. Preferred plants of the invention include gymnosperms, monocots, and dicots, especially agronomically important crop plants, such as rice, wheat, barley, rye, rape, corn, potato, carrot, sweet potato, sugar beet, bean, pea, chicory, lettuce, cabbage, cauliflower, broccoli, turnip, radish, spinach, asparagus, onion, garlic, eggplant, pepper, celery, carrot, squash, pumpkin, zucchini, cucumber, apple, pear, quince, melon, plum, cherry, peach, nectarine, apricot, strawberry, grape, raspberry, blackberry, pineapple, avocado, papaya, mango, banana, soybean, tobacco, tomato, sorghum and sugarcane. The genetic properties engineered into the transgenic seeds and plants described above are passed on by sexual reproduction and can thus be maintained and propagated in progeny plants.

EXAMPLES

The invention is illustrated in further detail by the following detailed procedures, preparations, and examples. The examples are for illustration only, and are not to be construed as limiting the scope of the present invention. Standard recombinant DNA and molecular cloning techniques used here are well known in the art and are described by Sambrook, *et al.*, 1989; by T.J. Silhavy, M.L. Berman, and L.W. Enquist, 1984; and by Ausubel, F.M. *et al.,* 1987.

I. Isolation of NIM1 Interactors

Example 1: *NIM1* Two-Hybrid Screen

The *Arabidopsis NIM1* gene (Ryals *et al.,* 1997) is used as a bait in a yeast two-hybrid screen. The screen used is the *LexA*-based version of the two hybrid screen originally developed by Fields and Song (1989) and later modified by Gyuris *et al.* (1993). A full length *NIM1* gene sequence is cloned into the bait plasmid pEG202 (Gyuris *et al.,* 1993) as a fusion with the *LexA* DNA binding domain using a PCR product (primers *NIM5*'RI and *NIM3*'SalI, see Table 1 below). Another bait (*NIM*450) is constructed using a nucleotide sequence encoding the first 450 amino acids of the NIM1 protein (Ryals *et al.,* 1997) with the primers *NIM* 5'RI and *NIM*trunc NCOI3'. A third bait containing only the C-terminal portion of the *NIM1* gene encoding amino acids 366-594 of the NIM1 protein (Ryals *et al.,* 1997) is cloned using the primers *NIM*loop5'RI and *NIM* 3'SalI. These baits are transformed into yeast strain EGY188, which contains a low sensitivity *LexA* operator fused to the *Leu2* gene, and appropriate tests are performed to determine whether the *NIM1* fusion proteins intrinsically activate expression of the yeast reporter genes. A yeast expression library (obtained from Jeff Dangl, UNC-Chapel Hill) is constructed in plasmid pJG4-5 using RNA from *Arabidopsis* leaves infected with *Pseudomonas syringae.* This library encodes fusion proteins of expressed *Arabidopsis* genes and the B42 transcriptional activation domain. The library is transformed in combination with one of the three *NIM1* baits above and a plasmid containing 2 *LexA* operators fused to *LacZ* as a secondary reporter. Approximately 10^6 transformants are

analyzed in screens with each bait, and individual clones that activate both the *Leu2* and *LacZ* reporters are isolated and sequenced by standard procedures. The most frequently isolated clone (NI16) is found 25 times with the *NIM*366-594 bait and 12 times with the full-length bait. The NI16 clone does not interact with the *NIM*450 bait.

A full-length NI16 cDNA is determined using a 5' RACE kit from BRL Life Technologies. cDNA primer 16GSP1 is used for CDNA synthesis. Then, following a TdT tailing reaction, primer 16GSP2 is used in combination with an abridged anchor primer provided by the manufacturer to amplify the cDNA product by PCR. A third primer, 16GSP3, is used as a PCR control in combination with the GSP2 primer. A cDNA fragment is amplified and cloned into the pCR2.1 vector (Clonetech) and sequenced.

The full-length *Arabidopsis* NI16 cDNA sequence is shown as SEQ ID NO:1, and the encoded 122-amino acid protein sequence is shown as SEQ ID NO:2. The NI16 gene sequence contains regions of high homology to the tobacco G8-1 gene, which is induced rapidly following treatment with salicylic acid, and which is responsive to very low concentrations of salicylate (Horvath et al., 1996).

Example 2: Induction of NI16 mRNA

1. Salicylic Acid and BTH Induce NI16 mRNA

RNA is prepared from 4 week old Wassilewskija (WS-O) plants, that are either sprayed with water, a 5mM solution of sodium salicylate (SA), or a 300μm solution of benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH). SA and BTH both have been demonstrated to activate SAR in *Arabidopsis* and to induce pathogenesis related (PR) gene expression by Ward *et al.* (1991) and Lawton *et al.* (1996). RNA extractions and RNA blot hybridizations are performed as described previously by Ausubel *et al.* (1987). NI16 RNA is induced 5-10 fold within15 minutes of SA or BTH induction and reaches a peak of approximately 50 fold induction within 2 hours of SA treatment and 25 fold induction with BTH treatment. The transcript remains highly induced up to 24 hours after SA and at least 48 hours after BTH treatment.

2. NI16 mRNA Is Induced In Response To Pathogen Infection And Is Not Induced In NahG or *nim1-4* Plants.

WS-O plants are infected with the avirulent pathogen *Pseudomonas syringae* p.v. *tomato* (P.s.t.) containing the *avrRPM1* gene as described previously by Lawton *et al.* (1995). Three leaves of four week old plants are inoculated on one-half of the midrib with either buffer or P.s.t. and local and systemic tissue are harvested at 0, 2, 4, 6, 12, 24, 48, and 72 hours. NI16 is induced 5 fold within 4 hours in local tissue, and 6 hours in systemic tissue, and remains induced up to 72 hours after infection. In a separate experiment, WS-0, *nim1-4*, and NahG plants are inoculated with the same P.s.t. isolate and systemic tissue is harvested after 48 hours. *nim1-4* plants are compromised in their ability to respond to pathogens as measured by their accumulation of PR-1 in response to chemical induction and pathogen treatment and their susceptibility to pathogens despite treatment with the chemical inducer 2,6-dichloroisonicotinic acid (Ryals *et al.*, 1997). NahG plants express the bacterial salicylate hydroxylase (*nahG)* gene and are unable to accumulate salicylic acid (Gaffney *et al.* (1993) and Delaney *et al.* (1994). In wild type WS-O plants, NI16 is induced approximately 4 fold following P.s.t. inoculation but is not induced in NahG plants and is induced only slightly in *nim1-4* plants. In another experiment *nim1-4* plants are unable to accumulate NI16 transcript after 2 and 24 hours of BTH treatment. It can be concluded from these experiments that salicylic acid and a functional NIM1 protein are necessary for induction of NI16 in response to pathogens.

Example 3: Genomic Cloning Of NI16 And Associated Promoter Sequence

A genomic copy of NI16 is cloned into the vector pBluescript from a Lambda ZapII library (Stratagene) according to the manufacturer's protocols. Two separate clones are obtained from the library. Clone 4-1 contains 3.1kb of sequence upstream of NI16 and the coding region of NI16 up to the internal *Eco*RI site (*see,* SEQ ID NO:1). A second clone, 5-1, contains sequence 3' to the *Eco*RI site and 3.1kb of sequence downstream of NI16. An intact genomic clone is obtained by first deleting a 1.87kb *Bgl*II/*Bam*HI fragment from clone 4-1 then ligating a 350bp *Eco*RI/*Eco*RV fragment from clone 5-1 into the remaining *Eco*RI and *Eco*RV sites of clone 4-1. The genomic clones are sequenced, producing the NI16 genomic

sequence shown as SEQ ID NO:3. Promoter elements are present in the 5' upstream region adjacent to the NI16 genomic coding region (*see*, SEQ ID NO:3). *E. coli* strain DH5α containing plasmid pRC25 (approximately 1.9kb *BglII*/*Eco*RV genomic NI16 fragment in pBluescript) has been deposited with the NRRL on Jan. 7, 2000, and assigned accession number NRRL B-30239.

Example 4: Transgenic Expression Of NI16 Results In Constitutive PR-1 Expression

A NI16 construct is made in binary vector pBAR35S (obtained from Jeff Dangl, UNC-Chapel Hill). First a full-length clone is obtained by cloning the 5'sequence of NI16 (obtained by 5' RACE and cloned into AT vector pCR2.1) into the *Eco*RI site of the original pJG4-5 clone as an *Eco*RI fragment. Then the entire coding region of NI16 is PCR cloned back into pCR2.1 with the primers 16F and 16 R (Table 1). The resulting plasmid is cut with *Xba*I and *Sac*I for cloning into pBAR35S. The clone is transformed into *Agrobacterium* strain GV3101 (Koncz and Schell, 1986). WS-O and *nim1-4* plants are transformed using standard procedures and the resulting T1 seed is selected by spraying with a 60μg/ml solution of BASTA every two days for 6 days after germination. BASTA resistant plants are analyzed for levels of NI16 and PR-1 RNA after 4 weeks.

4 WS-O plants expressing NI16 above wild type levels are recovered. All have elevated levels of PR-1 RNA as detected on Northern blots. All 4 plants are taken to the T3 generations. Quantitative Northerns are done on two of the T3 lines, one with a relatively low level of NI16 RNA (1-1B) and one with a high level of NI16 RNA (2-1B). Northerns are hybridized with NI16 and PR-1 probes. Measurements are taken with a Molecular Dynamics Phosphoimager, which directly measures counts per unit area. The amount of PR-1 RNA correlates directly with the amount of NI16 RNA, i.e., the highest expressers of NI16 have the highest levels of PR-1. (Table 2). BTH applications at rates lower than that normally used to provide protection to *Peronospora parasitica* do not significantly increase the amount of NI16 RNA in the transgenic lines. PR-1 levels are induced at the same rate in the two NI16 lines as the WS-O control, however steady state PR-1 levels are higher, especially at 0 and 10μm BTH. NI16 may therefore be necessary for the SAR response, and overexpression of NI16 may activate a subset of the response. PR-1 is also elevated in nahG ,*nim1*-1 and *nim1*-4

plants overexpressing NI16. Out of 34 *nim1*-1 T1 plants containing the 35S-NI16 construct, 22 have elevated levels of PR-1. Similarly, 18/26 *nim1*-4 and 20/27 nahG primary transformants have elevated PR-1 levels. In each case, high NI16 expression is correlated with high PR-1 levels. This is significant because *nim1* and nahG plants are normally deficient in their ability to induce PR1 in response to pathogens. Furthermore, this provides evidence that overexpression of NI16 can at least partially compensate for the absence of SA and a functional NIM protein in plants.

Example 5: Homologues Of NI16 Cloned By PCR From Potato and Tomato

Homologues of NI16 from potato and tomato are cloned using the Marathon cDNA amplification kit. cDNA is made from potato and tomato polyA RNA and linkers are ligated at both ends according to the manufacturer's instructions. Degenerate primers are designed by comparing the *Arabidopsis* NI16 sequence with the tobacco G8-1 sequence (Horvath *et al.*, 1998). The primers NI16 degR1 (SEQ ID NO:20) and NI16degF1 (SEQ ID NO:21), in combination with linker primers provided by Clonetech, are then used to amplify the 5' and 3' ends of the cDNAs. PCR products are then cloned using the TOPO-TA cloning kit from Invitrogen and sequenced. The entire potato cDNA (SEQ ID NO:4) and the 3'end of the tomato cDNA (SEQ ID NO:6) are recovered. Table 3 below shows a sequence alignment of the *Arabidopsis*, potato, and tomato clones, in addition to the polypeptide sequence encoded by the tobacco G8-1 gene and two soybean EST's (AI495102 and AI461039) identified by BLAST homology. All of the cDNA's encode small proteins (<122 amino acids) with a 22 amino acid core motif (bold) that is 45% identical and 82% similar among the 6 sequences.

Table 1. Primers Used In *NIM1* And NI16 Cloning

Primer Name	Sequence	SEQ ID NO:
NIM5'RI	ggaacgaattcatggacaccaccattg	SEQ ID NO:11
NIM3'SalI	aaaaaagtcgactaagagcaagagtc	SEQ ID NO:12
*NIM*trunc3'NcoI	cgatctccatggcagcttgtcc	SEQ ID NO:13
*NIM*loop5'RI	gaaccgaattcatgatcgca	SEQ ID NO:14
16GSP1	ttccggtttacagttcagat	SEQ ID NO:15
16GSP2	gacccgattaataatctcatcg	SEQ ID NO:16
16GSP3	caccatttctggttggaggt	SEQ ID NO:17
16F	acgacgccgttaacattttc	SEQ ID NO:18

16R	gaaggggaaaaacatgaagga	SEQ ID NO:19
NI16 DegF	cggaggnngaggtngaygagttyttc*	SEQ ID NO:20
NI16 DegR	gaaraactcrtcnacctcnnccctccg*	SEQ ID NO:21

*n = a, c, t, or g; y = c or t; r = a or g

Table 2. Relative levels of NI16 and PR-1 in wild type *Arabidopsis* and two NI16-overexpressing lines 24 hours after induction with 0, 10 or 100μm BTH.

Genotype	BTH conc.(μm)	NI16 level	PR-1 level
WS	0	1	1
	10	10	30
	100	90	90
1-1B	0	760	3.5
	10	1400	30
	100	1480	120
2-1B	0	11000	50
	10	14000	70
	100	12000	140

Table 3. Alignment of NI16 Homologous Sequences

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AI495102    ---MEVEKRKNKRVMGEEEESERVKNKRLKGVEEEDGSDGVPTEEEVEEFFAILRRMRMA 57
AI461039    ------------------------------------GGVPTEEEVEEFFAILRRMRVA 22
Potato      MLLMDGEKKRKRTAIG-------AGDRSKDEVEATVKEEEPPSEAEXDEFFAILRRMHVA 53
Tomato      ----------------------------------------SEGEVDEFFAILRRMHMA 18
G8-1        ---MDGEKKRKRTENGKANGGDRNRHERKSAANEHTAVSPPPSEAEVDEFFAILRRMHVA 57
NI16        ---MNNSLKKEERVEED------NGKSDGNRGKPSTEVVRTVTEEEVDEFFKILRRVHVA 51
                                                      :* * :*** ****:::*

AI495102    VKYFDDKGRGGRE-WREAL---------------------------------------- 75
AI461039    VKYFDDKGSGGKE-WRKALETAELTVDHRHDVVAAEEDDKPRKKGGEVI--INEGFDLNA 79
Potato      VKYLQRNAQIRPE-NLNAS-------PA-----GANGVAAGRKRERGIV--RKGDLDLNT 98
Tomato      VKYLQRNAQIQPE-NVNAHGSKLTASPA-----GVNGDATGQKRERGIV--RKGDLDLNT 70
G8-1        VRYLQESGQKR-------------------------VVP-----------KGDLDLNT 79
NI16        TRTVAKVNGGVAEGELPSKKRKRSQNLGLRNSLDCNGVRDGEFDEINRVGLQGLGLDLNC 111
            .: .                                                    ***
AI495102      -----------          (SEQ ID NO:8)
AI461039      VAPEAAEGGGA 90       (SEQ ID NO:9)
Potato        LPDGGD----- 104      (SEQ ID NO:5)
Tomato        LPDCGDT---- 77       (SEQ ID NO:7)
G8-1          LPGNGD----- 85       (SEQ ID NO:10)
NI16          KPEPDSVSLSL 122      (SEQ ID NO:2)
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Example 6: Isolation Of The NIM1 Interactor, NI19

In addition to the NI16 cDNA clone, the NI19 clone may also be isolated in the two-hybrid screen described in Example 1. A full-length clone is found be blasting the NI19 cDNA clone against the genbank database. A Bacterial Artificial Chromosome (BAC) in genbank (#F14J9) matches the sequence from bases 19726-19439. Additional flanking sequence (bases 19726-20000) is translated using GCG seqweb. An open reading frame is found between bases 19897-19513 that gives a predicted protein of 112 amino acids. The full-length *Arabidopsis* NI19 genomic sequence is shown as SEQ ID NO:22, and the encoded 112-amino acid protein sequence is shown as SEQ ID NO:23. Nucleotides 127-413 of SEQ ID NO:22 represent the original cDNA clone isolated in the NIM1 two-hybrid screen.

II. Expression of the Gene Sequences of the Invention In Plants

The NIM1 interactor of the present invention, NI16, can be incorporated into plant cells using conventional recombinant DNA technology. Generally, this involves inserting a coding sequence of the invention into an expression system to which the coding sequence is heterologous (i.e., not normally present) using standard cloning procedures known in the art. The vector contains the necessary elements for the transcription and translation of the inserted protein-coding sequences. A large number of vector systems known in the art can be used, such as plasmids, bacteriophage viruses and other modified viruses. Suitable vectors include, but are not limited to, viral vectors such as lambda vector systems λgtl1, λgtl0 and Charon 4; plasmid vectors such as pBI121, pBR322, pACYC177, pACYC184, pAR series, pKK223-3, pUC8, pUC9, pUC18, pUC19, pLG339, pRK290, pKC37, pKC101, pCDNAII; and other similar systems. The components of the expression system may also be modified to increase expression. For example, truncated sequences, nucleotide substitutions or other modifications may be employed. The expression systems described herein can be used to transform virtually any crop plant cell under suitable conditions. Transformed cells can be regenerated into whole plants such that the NIM1 interactor increases SAR gene (e.g., PR-1) expression in the transgenic plants.

Example 7: Construction of Plant Expression Cassettes

Coding sequences intended for expression in transgenic plants are first assembled in expression cassettes behind a suitable promoter expressible in plants. The expression cassettes may also comprise any further sequences required or selected for the expression of the transgene. Such sequences include, but are not restricted to, transcription terminators, extraneous sequences to enhance expression such as introns, vital sequences, and sequences intended for the targeting of the gene product to specific organelles and cell compartments. These expression cassettes can then be easily transferred to the plant transformation vectors described below. The following is a description of various components of typical expression cassettes.

1. Promoters

The selection of the promoter used in expression cassettes will determine the spatial and temporal expression pattern of the transgene in the transgenic plant. Selected promoters will express transgenes in specific cell types (such as leaf epidermal cells, mesophyll cells, root cortex cells) or in specific tissues or organs (roots, leaves or flowers, for example) and the selection will reflect the desired location of accumulation of the gene product. Alternatively, the selected promoter may drive expression of the gene under various inducing conditions. Promoters vary in their strength, i.e., ability to promote transcription. Depending upon the host cell system utilized, any one of a number of suitable promoters can be used, including the gene's native promoter. The following are non-limiting examples of promoters that may be used in expression cassettes.

a. Constitutive Expression, the Ubiquitin Promoter

Ubiquitin is a gene product known to accumulate in many cell types and its promoter has been cloned from several species for use in transgenic plants (*e.g.* sunflower - Binet *et al.* Plant Science 79: 87-94 (1991); maize - Christensen *et al.* Plant Molec. Biol. 12: 619-632 (1989); and *Arabidopsis* - Callis *et al., J. Biol. Chem.* 265:12486-12493 (1990) and Norris *et al., Plant Mol. Biol.* 21:895-906 (1993)). The maize ubiquitin promoter has been developed in transgenic monocot systems and its sequence and vectors constructed for monocot

transformation are disclosed in the patent publication EP 0 342 926 (to Lubrizol) which is herein incorporated by reference. Taylor *et al.* (Plant Cell Rep. 12: 491-495 (1993)) describe a vector (pAHC25) that comprises the maize ubiquitin promoter and first intron and its high activity in cell suspensions of numerous monocotyledons when introduced via microprojectile bombardment. The *Arabidopsis* ubiquitin promoter is ideal for use with the nucleotide sequences of the present invention. The ubiquitin promoter is suitable for gene expression in transgenic plants, both monocotyledons and dicotyledons. Suitable vectors are derivatives of pAHC25 or any of the transformation vectors described in this application, modified by the introduction of the appropriate ubiquitin promoter and/or intron sequences.

b. Constitutive Expression, the CaMV 35S Promoter

Construction of the plasmid pCGN1761 is described in the published patent application EP 0 392 225 (Example 23), which is hereby incorporated by reference. pCGN1761 contains the "double" CaMV 35S promoter and the *tml* transcriptional terminator with a unique *EcoRI* site between the promoter and the terminator and has a pUC-type backbone. A derivative of pCGN1761 is constructed which has a modified polylinker which includes *NotI* and *XhoI* sites in addition to the existing *EcoRI* site. This derivative is designated pCGN1761ENX. pCGN1761ENX is useful for the cloning of cDNA sequences or coding sequences (including microbial ORF sequences) within its polylinker for the purpose of their expression under the control of the 35S promoter in transgenic plants. The entire 35S promoter-coding sequence-*tml* terminator cassette of such a construction can be excised by *HindIII, SphI, SalI,* and *XbaI* sites 5' to the promoter and *XbaI, BamHI* and *BglI* sites 3' to the terminator for transfer to transformation vectors such as those described below. Furthermore, the double 35S promoter fragment can be removed by 5' excision with *HindIII, SphI, SalI, XbaI*, or *PstI*, and 3' excision with any of the polylinker restriction sites (*EcoRI, NotI* or *XhoI*) for replacement with another promoter. If desired, modifications around the cloning sites can be made by the introduction of sequences that may enhance translation. This is particularly useful when overexpression is desired. For example, pCGN1761ENX may be modified by optimization of the translational initiation site as described in Example 37 of U.S. Patent No. 5,639,949, incorporated herein by reference.

c. Constitutive Expression, the Actin Promoter

Several isoforms of actin are known to be expressed in most cell types and consequently the actin promoter is a good choice for a constitutive promoter. In particular, the promoter from the rice *ActI* gene has been cloned and characterized (McElroy *et al.* Plant Cell 2: 163-171 (1990)). A 1.3kb fragment of the promoter was found to contain all the regulatory elements required for expression in rice protoplasts. Furthermore, numerous expression vectors based on the *ActI* promoter have been constructed specifically for use in monocotyledons (McElroy *et al.* Mol. Gen. Genet. 231: 150-160 (1991)). These incorporate the *ActI*-intron 1, *AdhI* 5' flanking sequence and *AdhI*-intron 1 (from the maize alcohol dehydrogenase gene) and sequence from the CaMV 35S promoter. Vectors showing highest expression were fusions of 35S and *ActI* intron or the *ActI* 5' flanking sequence and the *ActI* intron. Optimization of sequences around the initiating ATG (of the GUS reporter gene) also enhanced expression. The promoter expression cassettes described by McElroy *et al.* (Mol. Gen. Genet. 231: 150-160 (1991)) can be easily modified for gene expression and are particularly suitable for use in monocotyledonous hosts. For example, promoter-containing fragments is removed from the McElroy constructions and used to replace the double 35S promoter in pCGN1761ENX, which is then available for the insertion of specific gene sequences. The fusion genes thus constructed can then be transferred to appropriate transformation vectors. In a separate report, the rice *ActI* promoter with its first intron has also been found to direct high expression in cultured barley cells (Chibbar *et al.* Plant Cell Rep. 12: 506-509 (1993)).

d. Inducible Expression, PR-1 Promoters

The double 35S promoter in pCGN1761ENX may be replaced with any other promoter of choice that will result in suitably high expression levels. By way of example, one of the chemically regulatable promoters described in U.S. Patent No. 5,614,395, such as the tobacco PR-1a promoter, may replace the double 35S promoter. Alternately, the *Arabidopsis* PR-1 promoter described in Lebel *et al., Plant J.* 16:223-233 (1998) may be used. The promoter of choice is preferably excised from its source by restriction enzymes, but can alternatively be PCR-amplified using primers that carry appropriate terminal restriction sites. Should PCR-amplification be undertaken, then the promoter should be re-sequenced to check for

amplification errors after the cloning of the amplified promoter in the target vector. The chemically/pathogen regulatable tobacco PR-1a promoter is cleaved from plasmid pCIB1004 (for construction, see example 21 of EP 0 332 104, which is hereby incorporated by reference) and transferred to plasmid pCGN1761ENX (Uknes *et al.*, *Plant Cell* 4: 645-656 (1992)). pCIB1004 is cleaved with *NcoI* and the resultant 3' overhang of the linearized fragment is rendered blunt by treatment with T4 DNA polymerase. The fragment is then cleaved with *HindIII* and the resultant PR-1a promoter-containing fragment is gel purified and cloned into pCGN1761ENX from which the double 35S promoter has been removed. This is done by cleavage with *XhoI* and blunting with T4 polymerase, followed by cleavage with *HindIII* and isolation of the larger vector-terminator containing fragment into which the pCIB1004 promoter fragment is cloned. This generates a pCGN1761ENX derivative with the PR-1a promoter and the *tml* terminator and an intervening polylinker with unique *EcoRI* and *NotI* sites. The selected coding sequence can be inserted into this vector, and the fusion products (*i.e.* promoter-gene-terminator) can subsequently be transferred to any selected transformation vector, including those described *infra*. Various chemical regulators may be employed to induce expression of the selected coding sequence in the plants transformed according to the present invention, including the benzothiadiazole, isonicotinic acid, and salicylic acid compounds disclosed in U.S. Patent Nos. 5,523,311 and 5,614,395.

e. Inducible Expression, an Ethanol-Inducible Promoter

A promoter inducible by certain alcohols or ketones, such as ethanol, may also be used to confer inducible expression of a coding sequence of the present invention. Such a promoter is for example the *alcA* gene promoter from *Aspergillus nidulans* (Caddick et al. (1998) *Nat. Biotechnol* 16:177-180). In *A. nidulans*, the *alcA* gene encodes alcohol dehydrogenase I, the expression of which is regulated by the AlcR transcription factors in presence of the chemical inducer. For the purposes of the present invention, the CAT coding sequences in plasmid palcA:CAT comprising a *alcA* gene promoter sequence fused to a minimal 35S promoter (Caddick et al. (1998) *Nat. Biotechnol* 16:177-180) are replaced by a coding sequence of the present invention to form an expression cassette having the coding sequence under the control of the *alcA* gene promoter. This is carried out using methods well known in the art.

f. Inducible Expression, a Glucocorticoid-Inducible Promoter

Induction of expression of a nucleic acid sequence of the present invention using systems based on steroid hormones is also contemplated. For example, a glucocorticoid-mediated induction system is used (Aoyama and Chua (1997) *The Plant Journal* 11: 605-612) and gene expression is induced by application of a glucocorticoid, for example a synthetic glucocorticoid, preferably dexamethasone, preferably at a concentration ranging from 0.1mM to 1mM, more preferably from 10mM to 100mM. For the purposes of the present invention, the luciferase gene sequences are replaced by a nucleic acid sequence of the invention to form an expression cassette having a nucleic acid sequence of the invention under the control of six copies of the GAL4 upstream activating sequences fused to the 35S minimal promoter. This is carried out using methods well known in the art. The trans-acting factor comprises the GAL4 DNA-binding domain (Keegan et al. (1986) *Science* 231: 699-704) fused to the transactivating domain of the herpes viral protein VP16 (Triezenberg et al. (1988) *Genes Devel.* 2: 718-729) fused to the hormone-binding domain of the rat glucocorticoid receptor (Picard et al. (1988) *Cell* 54: 1073-1080). The expression of the fusion protein is controlled by any promoter suitable for expression in plants known in the art or described here. This expression cassette is also comprised in the plant comprising a nucleic acid sequence of the invention fused to the 6xGAL4/minimal promoter. Thus, tissue- or organ-specificity of the fusion protein is achieved leading to inducible tissue- or organ-specificity of the insecticidal toxin.

g. Root Specific Expression

Another pattern of gene expression is root expression. A suitable root promoter is the promoter of the maize metallothionein-like (MTL) gene described by de Framond (FEBS 290: 103-106 (1991)) and also in U.S. Patent No. 5,466,785, incorporated herein by reference. This "MTL" promoter is transferred to a suitable vector such as pCGN1761ENX for the insertion of a selected gene and subsequent transfer of the entire promoter-gene-terminator cassette to a transformation vector of interest.

h. Wound-Inducible Promoters

Wound-inducible promoters may also be suitable for gene expression. Numerous such promoters have been described (*e.g.* Xu *et al.* Plant Molec. Biol. 22: 573-588 (1993),

Logemann *et al.* Plant Cell 1: 151-158 (1989), Rohrmeier & Lehle, Plant Molec. Biol. 22: 783-792 (1993), Firek *et al.* Plant Molec. Biol. 22: 129-142 (1993), Warner *et al.* Plant J. 3: 191-201 (1993)) and all are suitable for use with the instant invention. Logemann *et al.* describe the 5' upstream sequences of the dicotyledonous potato *wunI* gene. Xu *et al.* show that a wound-inducible promoter from the dicotyledon potato (*pin2*) is active in the monocotyledon rice. Further, Rohrmeier & Lehle describe the cloning of the maize *WipI* cDNA which is wound induced and which can be used to isolate the cognate promoter using standard techniques. Similar, Firek *et al.* and Warner *et al.* have described a wound-induced gene from the monocotyledon *Asparagus officinalis,* which is expressed at local wound and pathogen invasion sites. Using cloning techniques well known in the art, these promoters can be transferred to suitable vectors, fused to the genes pertaining to this invention, and used to express these genes at the sites of plant wounding.

i. Pith-Preferred Expression

Patent Application WO 93/07278, which is herein incorporated by reference, describes the isolation of the maize *trpA* gene, which is preferentially expressed in pith cells. The gene sequence and promoter extending up to -1726 bp from the start of transcription are presented. Using standard molecular biological techniques, this promoter, or parts thereof, can be transferred to a vector such as pCGN1761 where it can replace the 35S promoter and be used to drive the expression of a foreign gene in a pith-preferred manner. In fact, fragments containing the pith-preferred promoter or parts thereof can be transferred to any vector and modified for utility in transgenic plants.

j. Leaf-Specific Expression

A maize gene encoding phosphoenol carboxylase (PEPC) has been described by Hudspeth & Grula (Plant Molec Biol 12: 579-589 (1989)). Using standard molecular biological techniques the promoter for this gene can be used to drive the expression of any gene in a leaf-specific manner in transgenic plants.

k. Pollen-Specific Expression

WO 93/07278 describes the isolation of the maize calcium-dependent protein kinase (CDPK) gene which is expressed in pollen cells. The gene sequence and promoter extend up to 1400 bp from the start of transcription. Using standard molecular biological techniques, this promoter or parts thereof, can be transferred to a vector such as pCGN1761 where it can replace the 35S promoter and be used to drive the expression of a nucleic acid sequence of the invention in a pollen-specific manner.

2. Transcriptional Terminators

A variety of transcriptional terminators are available for use in expression cassettes. These are responsible for the termination of transcription beyond the transgene and correct mRNA polyadenylation. Appropriate transcriptional terminators are those that are known to function in plants and include the CaMV 35S terminator, the *tml* terminator, the nopaline synthase terminator and the pea *rbcS* E9 terminator. These can be used in both monocotyledons and dicotyledons. In addition, a gene's native transcription terminator may be used.

3. Sequences for the Enhancement or Regulation of Expression

Numerous sequences have been found to enhance gene expression from within the transcriptional unit and these sequences can be used in conjunction with the genes of this invention to increase their expression in transgenic plants.

Various intron sequences have been shown to enhance expression, particularly in monocotyledonous cells. For example, the introns of the maize *AdhI* gene have been found to significantly enhance the expression of the wild-type gene under its cognate promoter when introduced into maize cells. Intron 1 was found to be particularly effective and enhanced expression in fusion constructs with the chloramphenicol acetyltransferase gene (Callis *et al.*, *Genes Develop.* 1: 1183-1200 (1987)). In the same experimental system, the intron from the maize *bronze1* gene had a similar effect in enhancing expression. Intron sequences have been routinely incorporated into plant transformation vectors, typically within the non-translated leader.

A number of non-translated leader sequences derived from viruses are also known to enhance expression, and these are particularly effective in dicotyledonous cells. Specifically, leader sequences from Tobacco Mosaic Virus (TMV, the "W-sequence"), Maize Chlorotic Mottle Virus (MCMV), and Alfalfa Mosaic Virus (AMV) have been shown to be effective in enhancing expression (*e.g.* Gallie *et al. Nucl. Acids Res.* 15: 8693-8711 (1987); Skuzeski *et al. Plant Molec. Biol.* 15: 65-79 (1990)). Other leader sequences known in the art include but are not limited to: picornavirus leaders, for example, EMCV leader (Encephalomyocarditis 5' noncoding region) (Elroy-Stein, O., Fuerst, T. R., and Moss, B. *PNAS USA* 86:6126-6130 (1989)); potyvirus leaders, for example, TEV leader (Tobacco Etch Virus) (Allison et al., 1986); MDMV leader (Maize Dwarf Mosaic Virus); *Virology* 154:9-20); human immunoglobulin heavy-chain binding protein (BiP) leader, (Macejak, D. G., and Samow, P., *Nature* 353: 90-94 (1991); untranslated leader from the coat protein mRNA of alfalfa mosaic virus (AMV RNA 4), (Jobling, S. A., and Gehrke, L., *Nature* 325:622-625 (1987); tobacco mosaic virus leader (TMV), (Gallie, D. R. et al., *Molecular Biology of RNA*, pages 237-256 (1989); and Maize Chlorotic Mottle Virus leader (MCMV) (Lommel, S. A. et al., *Virology* 81:382-385 (1991). See also, Della-Cioppa et al., *Plant Physiology* 84:965-968 (1987).

In addition to incorporating one or more of the aforementioned elements into the 5' regulatory region of a target expression cassette of the invention, other elements peculiar to the target expression cassette may also be incorporated. Such elements include but are not limited to a minimal promoter. By minimal promoter it is intended that the basal promoter elements are inactive or nearly so without upstream activation. Such a promoter has low background activity in plants when there is no transactivator present or when enhancer or response element binding sites are absent. One minimal promoter that is particularly useful for target genes in plants is the Bz1 minimal promoter, which is obtained from the bronze1 gene of maize. The Bz1 core promoter is obtained from the "myc" mutant Bz1-luciferase construct pBz1LucR98 via cleavage at the NheI site located at -53 to -58. Roth *et al., Plant Cell* 3: 317 (1991). The derived Bz1 core promoter fragment thus extends from -53 to +227 and includes the Bz1 intron-1 in the 5' untranslated region. Also useful for the invention is a minimal promoter created by use of a synthetic TATA element. The TATA element allows recognition of the promoter by RNA polymerase factors and confers a basal level of gene expression in

the absence of activation (*see generally*, Mukumoto (1993) *Plant Mol Biol* 23: 995-1003; Green (2000) *Trends Biochem Sci* 25: 59-63)

4. Targeting of the Gene Product Within the Cell

Various mechanisms for targeting gene products are known to exist in plants and the sequences controlling the functioning of these mechanisms have been characterized in some detail. For example, the targeting of gene products to the chloroplast is controlled by a signal sequence found at the amino terminal end of various proteins which is cleaved during chloroplast import to yield the mature protein (*e.g.* Comai *et al.* J. Biol. Chem. 263: 15104-15109 (1988)). These signal sequences can be fused to heterologous gene products to effect the import of heterologous products into the chloroplast (van den Broeck, et al. Nature 313: 358-363 (1985)). DNA encoding for appropriate signal sequences can be isolated from the 5' end of the cDNAs encoding the RUBISCO protein, the CAB protein, the EPSP synthase enzyme, the GS2 protein and many other proteins which are known to be chloroplast localized. *See also*, the section entitled "Expression With Chloroplast Targeting" in Example 37 of U.S. Patent No. 5,639,949.

Other gene products are localized to other organelles such as the mitochondrion and the peroxisome (*e.g.* Unger *et al.* Plant Molec. Biol. 13: 411-418 (1989)). The cDNAs encoding these products can also be manipulated to effect the targeting of heterologous gene products to these organelles. Examples of such sequences are the nuclear-encoded ATPases and specific aspartate amino transferase isoforms for mitochondria. Targeting cellular protein bodies has been described by Rogers *et al.* (Proc. Natl. Acad. Sci. USA 82: 6512-6516 (1985)).

In addition, sequences have been characterized which cause the targeting of gene products to other cell compartments. Amino terminal sequences are responsible for targeting to the ER, the apoplast, and extracellular secretion from aleurone cells (Koehler & Ho, Plant Cell 2: 769-783 (1990)). Additionally, amino terminal sequences in conjunction with carboxy terminal sequences are responsible for vacuolar targeting of gene products (Shinshi *et al.* Plant Molec. Biol. 14: 357-368 (1990)).

By the fusion of the appropriate targeting sequences described above to transgene sequences of interest it is possible to direct the transgene product to any organelle or cell compartment. For chloroplast targeting, for example, the chloroplast signal sequence from the

RUBISCO gene, the CAB gene, the EPSP synthase gene, or the GS2 gene is fused in frame to the amino terminal ATG of the transgene. The signal sequence selected should include the known cleavage site, and the fusion constructed should take into account any amino acids after the cleavage site which are required for cleavage. In some cases this requirement may be fulfilled by the addition of a small number of amino acids between the cleavage site and the transgene ATG or, alternatively, replacement of some amino acids within the transgene sequence. Fusions constructed for chloroplast import can be tested for efficacy of chloroplast uptake by *in vitro* translation of *in vitro* transcribed constructions followed by *in vitro* chloroplast uptake using techniques described by Bartlett *et al.* In: Edelmann *et al.* (Eds.) Methods in Chloroplast Molecular Biology, Elsevier pp 1081-1091 (1982) and Wasmann *et al.* Mol. Gen. Genet. 205: 446-453 (1986). These construction techniques are well known in the art and are equally applicable to mitochondria and peroxisomes.

The above-described mechanisms for cellular targeting can be utilized not only in conjunction with their cognate promoters, but also in conjunction with heterologous promoters so as to effect a specific cell-targeting goal under the transcriptional regulation of a promoter that has an expression pattern different to that of the promoter from which the targeting signal derives.

Example 8: Construction of Plant Transformation Vectors

Numerous transformation vectors available for plant transformation are known to those of ordinary skill in the plant transformation arts, and the genes pertinent to this invention can be used in conjunction with any such vectors. The selection of vector will depend upon the preferred transformation technique and the target species for transformation. For certain target species, different antibiotic or herbicide selection markers may be preferred. Selection markers used routinely in transformation include the *nptII* gene, which confers resistance to kanamycin and related antibiotics (Messing & Vierra. Gene 19: 259-268 (1982); Bevan et al., Nature 304:184-187 (1983)), the *bar* gene, which confers resistance to the herbicide phosphinothricin (White et al., Nucl. Acids Res 18: 1062 (1990), Spencer et al. Theor. Appl. Genet 79: 625-631 (1990)), the *hph* gene, which confers resistance to the antibiotic hygromycin (Blochinger & Diggelmann, Mol Cell Biol 4: 2929-2931), and the *dhfr* gene,

which confers resistance to methatrexate (Bourouis et al., EMBO J. 2(7): 1099-1104 (1983)), the EPSPS gene, which confers resistance to glyphosate (U.S. Patent Nos. 4,940,935 and 5,188,642), and the mannose-6-phosphate isomerase gene, which provides the ability to metabolize mannose (U.S. Patent Nos. 5,767,378 and 5,994,629).

1. Vectors Suitable for *Agrobacterium* Transformation

Many vectors are available for transformation using *Agrobacterium tumefaciens*. These typically carry at least one T-DNA border sequence and include vectors such as pBIN19 (Bevan, Nucl. Acids Res. (1984)). Below, the construction of two typical vectors suitable for *Agrobacterium* transformation is described.

a. pCIB200 and pCIB2001:

The binary vectors pCIB200 and pCIB2001 are used for the construction of recombinant vectors for use with *Agrobacterium* and are constructed in the following manner. pTJS75kan is created by *NarI* digestion of pTJS75 (Schmidhauser & Helinski, J. Bacteriol. 164: 446-455 (1985)) allowing excision of the tetracycline-resistance gene, followed by insertion of an *AccI* fragment from pUC4K carrying an NPTII (Messing & Vierra, Gene 19: 259-268 (1982): Bevan et al., Nature 304: 184-187 (1983): McBride et al., Plant Molecular Biology 14: 266-276 (1990)). *XhoI* linkers are ligated to the *EcoRV* fragment of PCIB7 which contains the left and right T-DNA borders, a plant selectable *nos/nptII* chimeric gene and the pUC polylinker (Rothstein et al., Gene 53: 153-161 (1987)), and the *Xhol*-digested fragment are cloned into *SalI*-digested pTJS75kan to create pCIB200 (see also EP 0 332 104, example 19). pCIB200 contains the following unique polylinker restriction sites: *EcoRI, SstI, KpnI, BglII, XbaI,* and *SalI.* pCIB2001 is a derivative of pCIB200 created by the insertion into the polylinker of additional restriction sites. Unique restriction sites in the polylinker of pCIB2001 are *EcoRI, SstI, KpnI, BglII, XbaI, SalI, MluI, BclI, AvrII, ApaI, HpaI,* and *StuI.* pCIB2001, in addition to containing these unique restriction sites also has plant and bacterial kanamycin selection, left and right T-DNA borders for *Agrobacterium*-mediated transformation, the RK2-derived *trfA* function for mobilization between *E. coli* and other hosts, and the *OriT* and *OriV* functions also from RK2. The pCIB2001 polylinker is suitable for the cloning of plant expression cassettes containing their own regulatory signals.

b. pCIB10 and Hygromycin Selection Derivatives thereof:

The binary vector pCIB10 contains a gene encoding kanamycin resistance for selection in plants and T-DNA right and left border sequences and incorporates sequences from the wide host-range plasmid pRK252 allowing it to replicate in both *E. coli* and *Agrobacterium*. Its construction is described by Rothstein *et al.* (Gene 53: 153-161 (1987)). Various derivatives of pCIB10 are constructed which incorporate the gene for hygromycin B phosphotransferase described by Gritz *et al.* (Gene 25: 179-188 (1983)). These derivatives enable selection of transgenic plant cells on hygromycin only (pCIB743), or hygromycin and kanamycin (pCIB715, pCIB717).

2. Vectors Suitable for non-*Agrobacterium* Transformation

Transformation without the use of *Agrobacterium tumefaciens* circumvents the requirement for T-DNA sequences in the chosen transformation vector and consequently vectors lacking these sequences can be utilized in addition to vectors such as the ones described above which contain T-DNA sequences. Transformation techniques that do not rely on *Agrobacterium* include transformation via particle bombardment, protoplast uptake (*e.g.* PEG and electroporation) and microinjection. The choice of vector depends largely on the preferred selection for the species being transformed. Below, the construction of typical vectors suitable for non-*Agrobacterium* transformation is described.

a. pCIB3064:

pCIB3064 is a pUC-derived vector suitable for direct gene transfer techniques in combination with selection by the herbicide basta (or phosphinothricin). The plasmid pCIB246 comprises the CaMV 35S promoter in operational fusion to the *E. coli* GUS gene and the CaMV 35S transcriptional terminator and is described in the PCT published application WO 93/07278. The 35S promoter of this vector contains two ATG sequences 5' of the start site. These sites are mutated using standard PCR techniques in such a way as to remove the ATGs and generate the restriction sites *SspI* and *PvuII*. The new restriction sites are 96 and 37 bp away from the unique *SalI* site and 101 and 42 bp away from the actual start site. The resultant derivative of pCIB246 is designated pCIB3025. The GUS gene is then

excised from pCIB3025 by digestion with *SalI* and *SacI*, the termini rendered blunt and religated to generate plasmid pCIB3060. The plasmid pJIT82 is obtained from the John Innes Centre, Norwich and the a 400 bp *SmaI* fragment containing the *bar* gene from *Streptomyces viridochromogenes* is excised and inserted into the *HpaI* site of pCIB3060 (Thompson *et al.* EMBO J 6: 2519-2523 (1987)). This generated pCIB3064, which comprises the *bar* gene under the control of the CaMV 35S promoter and terminator for herbicide selection, a gene for ampicillin resistance (for selection in *E. coli*) and a polylinker with the unique sites *SphI, PstI, HindIII,* and *BamHI.* This vector is suitable for the cloning of plant expression cassettes containing their own regulatory signals.

b. pSOG19 and pSOG35:

pSOG35 is a transformation vector that utilizes the *E. coli* gene dihydrofolate reductase (DFR) as a selectable marker conferring resistance to methotrexate. PCR is used to amplify the 35S promoter (-800 bp), intron 6 from the maize Adh1 gene (-550 bp) and 18 bp of the GUS untranslated leader sequence from pSOG10. A 250-bp fragment encoding the *E. coli* dihydrofolate reductase type II gene is also amplified by PCR and these two PCR fragments are assembled with a *SacI-PstI* fragment from pB1221 (Clontech) which comprises the pUC19 vector backbone and the nopaline synthase terminator. Assembly of these fragments generates pSOG19 which contains the 35S promoter in fusion with the intron 6 sequence, the GUS leader, the DHFR gene and the nopaline synthase terminator. Replacement of the GUS leader in pSOG19 with the leader sequence from Maize Chlorotic Mottle Virus (MCMV) generates the vector pSOG35. pSOG19 and pSOG35 carry the pUC gene for ampicillin resistance and have *HindIII, SphI, PstI* and *EcoRI* sites available for the cloning of foreign substances.

3. Vector Suitable for Chloroplast Transformation

For expression of a nucleotide sequence of the present invention in plant plastids, plastid transformation vector pPH143 (WO 97/32011, example 36) is used. The nucleotide sequence is inserted into pPH143 thereby replacing the PROTOX coding sequence. This vector is then used for plastid transformation and selection of transformants for spectinomycin

resistance. Alternatively, the nucleotide sequence is inserted in pPH143 so that it replaces the aadH gene. In this case, transformants are selected for resistance to PROTOX inhibitors.

Example 9: Transformation

Once a nucleic acid sequence of the invention has been cloned into an expression system, it is transformed into a plant cell. The receptor and target expression cassettes of the present invention can be introduced into the plant cell in a number of art-recognized ways. Methods for regeneration of plants are also well known in the art. For example, Ti plasmid vectors have been utilized for the delivery of foreign DNA, as well as direct DNA uptake, liposomes, electroporation, microinjection, and microprojectiles. In addition, bacteria from the genus *Agrobacterium* can be utilized to transform plant cells. Below are descriptions of representative techniques for transforming both dicotyledonous and monocotyledonous plants, as well as a representative plastid transformation technique.

1. Transformation of Dicotyledons

Transformation techniques for dicotyledons are well known in the art and include *Agrobacterium*-based techniques and techniques that do not require *Agrobacterium*. Non-*Agrobacterium* techniques involve the uptake of exogenous genetic material directly by protoplasts or cells. This can be accomplished by PEG or electroporation mediated uptake, particle bombardment-mediated delivery, or microinjection. Examples of these techniques are described by Paszkowski *et al.*, EMBO J 3: 2717-2722 (1984), Potrykus *et al.*, Mol. Gen. Genet. 199: 169-177 (1985), Reich *et al.*, Biotechnology 4: 1001-1004 (1986), and Klein *et al.*, Nature 327: 70-73 (1987). In each case the transformed cells are regenerated to whole plants using standard techniques known in the art.

Agrobacterium-mediated transformation is a preferred technique for transformation of dicotyledons because of its high efficiency of transformation and its broad utility with many different species. *Agrobacterium* transformation typically involves the transfer of the binary vector carrying the foreign DNA of interest (*e.g.* pCIB200 or pCIB2001) to an appropriate *Agrobacterium* strain which may depend of the complement of *vir* genes carried by the host *Agrobacterium* strain either on a co-resident Ti plasmid or chromosomally (*e.g.* strain CIB542

for pCIB200 and pCIB2001 (Uknes *et al.* Plant Cell 5: 159-169 (1993)). The transfer of the recombinant binary vector to *Agrobacterium* is accomplished by a triparental mating procedure using *E. coli* carrying the recombinant binary vector, a helper *E. coli* strain which carries a plasmid such as pRK2013 and which is able to mobilize the recombinant binary vector to the target *Agrobacterium* strain. Alternatively, the recombinant binary vector can be transferred to *Agrobacterium* by DNA transformation (Höfgen & Willmitzer, Nucl. Acids Res. 16: 9877 (1988)).

Transformation of the target plant species by recombinant *Agrobacterium* usually involves co-cultivation of the *Agrobacterium* with explants from the plant and follows protocols well known in the art. Transformed tissue is regenerated on selectable medium carrying the antibiotic or herbicide resistance marker present between the binary plasmid T-DNA borders.

Another approach to transforming plant cells with a gene involves propelling inert or biologically active particles at plant tissues and cells. This technique is disclosed in U.S. Patent Nos. 4,945,050, 5,036,006, and 5,100,792 all to Sanford et al. Generally, this procedure involves propelling inert or biologically active particles at the cells under conditions effective to penetrate the outer surface of the cell and afford incorporation within the interior thereof. When inert particles are utilized, the vector can be introduced into the cell by coating the particles with the vector containing the desired gene. Alternatively, the target cell can be surrounded by the vector so that the vector is carried into the cell by the wake of the particle. Biologically active particles (e.g., dried yeast cells, dried bacterium or a bacteriophage, each containing DNA sought to be introduced) can also be propelled into plant cell tissue.

2. Transformation of Monocotyledons

Transformation of most monocotyledon species has now also become routine. Preferred techniques include direct gene transfer into protoplasts using PEG or electroporation techniques, and particle bombardment into callus tissue. Transformations can be undertaken with a single DNA species or multiple DNA species (*i.e.* co-transformation) and both these techniques are suitable for use with this invention. Co-transformation may have the advantage of avoiding complete vector construction and of generating transgenic plants with unlinked

loci for the gene of interest and the selectable marker, enabling the removal of the selectable marker in subsequent generations, should this be regarded desirable. However, a disadvantage of the use of co-transformation is the less than 100% frequency with which separate DNA species are integrated into the genome (Schocher *et al.* Biotechnology 4: 1093-1096 (1986)).

Patent Applications EP 0 292 435, EP 0 392 225, and WO 93/07278 describe techniques for the preparation of callus and protoplasts from an elite inbred line of maize, transformation of protoplasts using PEG or electroporation, and the regeneration of maize plants from transformed protoplasts. Gordon-Kamm *et al.* (Plant Cell 2: 603-618 (1990)) and Fromm *et al.* (Biotechnology 8: 833-839 (1990)) have published techniques for transformation of A188-derived maize line using particle bombardment. Furthermore, WO 93/07278 and Koziel *et al.* (Biotechnology 11: 194-200 (1993)) describe techniques for the transformation of elite inbred lines of maize by particle bombardment. This technique utilizes immature maize embryos of 1.5-2.5 mm length excised from a maize ear 14-15 days after pollination and a PDS-1000He Biolistics device for bombardment.

Transformation of rice can also be undertaken by direct gene transfer techniques utilizing protoplasts or particle bombardment. Protoplast-mediated transformation has been described for *Japonica*-types and *Indica*-types (Zhang *et al.* Plant Cell Rep 7: 379-384 (1988); Shimamoto *et al.* Nature 338: 274-277 (1989); Datta *et al.* Biotechnology 8: 736-740 (1990)). Both types are also routinely transformable using particle bombardment (Christou *et al.* Biotechnology 9: 957-962 (1991)). Furthermore, WO 93/21335 describes techniques for the transformation of rice via electroporation.

Patent Application EP 0 332 581 describes techniques for the generation, transformation and regeneration of Pooideae protoplasts. These techniques allow the transformation of *Dactylis* and wheat. Furthermore, wheat transformation has been described by Vasil *et al.* (Biotechnology 10: 667-674 (1992)) using particle bombardment into cells of type C long-term regenerable callus, and also by Vasil *et al.* (Biotechnology 11: 1553-1558 (1993)) and Weeks *et al.* (Plant Physiol. 102: 1077-1084 (1993)) using particle bombardment of immature embryos and immature embryo-derived callus. A preferred technique for wheat transformation, however, involves the transformation of wheat by particle bombardment of immature embryos and includes either a high sucrose or a high maltose step prior to gene delivery. Prior to bombardment, any number of embryos (0.75-1 mm in length) are plated

onto MS medium with 3% sucrose (Murashiga & Skoog, Physiologia Plantarum 15: 473-497 (1962)) and 3 mg/l 2,4-D for induction of somatic embryos, which is allowed to proceed in the dark. On the chosen day of bombardment, embryos are removed from the induction medium and placed onto the osmoticum (*i.e.* induction medium with sucrose or maltose added at the desired concentration, typically 15%). The embryos are allowed to plasmolyze for 2-3 hours and are then bombarded. Twenty embryos per target plate is typical, although not critical. An appropriate gene-carrying plasmid (such as pCIB3064 or pSG35) is precipitated onto micrometer size gold particles using standard procedures. Each plate of embryos is shot with the DuPont Biolistics® helium device using a burst pressure of ~1000 psi using a standard 80 mesh screen. After bombardment, the embryos are placed back into the dark to recover for about 24 hours (still on osmoticum). After 24 hrs, the embryos are removed from the osmoticum and placed back onto induction medium where they stay for about a month before regeneration. Approximately one month later the embryo explants with developing embryogenic callus are transferred to regeneration medium (MS + 1 mg/liter NAA, 5 mg/liter GA), further containing the appropriate selection agent (10 mg/l basta in the case of pCIB3064 and 2 mg/l methotrexate in the case of pSOG35). After approximately one month, developed shoots are transferred to larger sterile containers known as "GA7s" which contain half-strength MS, 2% sucrose, and the same concentration of selection agent.

Tranformation of monocotyledons using *Agrobacterium* has also been described. *See,* WO 94/00977 and U.S. Patent No. 5,591,616, both of which are incorporated herein by reference.

3. Transformation of Plastids

Seeds of *Nicotiana tabacum* c.v. 'Xanthi nc' are germinated seven per plate in a 1" circular array on T agar medium and bombarded 12-14 days after sowing with 1 μm tungsten particles (M10, Biorad, Hercules, CA) coated with DNA from plasmids pPH143 and pPH145 essentially as described (Svab, Z. and Maliga, P. (1993) *PNAS* 90, 913–917). Bombarded seedlings are incubated on T medium for two days after which leaves are excised and placed abaxial side up in bright light (350-500 µmol photons/m^2/s) on plates of RMOP medium (Svab, Z., Hajdukiewicz, P. and Maliga, P. (1990) *PNAS* 87, 8526–8530) containing 500 µg/ml spectinomycin dihydrochloride (Sigma, St. Louis, MO). Resistant shoots appearing

underneath the bleached leaves three to eight weeks after bombardment are subcloned onto the same selective medium, allowed to form callus, and secondary shoots isolated and subcloned. Complete segregation of transformed plastid genome copies (homoplasmicity) in independent subclones is assessed by standard techniques of Southern blotting (Sambrook et al., (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor). BamHI/EcoRI-digested total cellular DNA (Mettler, I. J. (1987) *Plant Mol Biol Reporter* 5, 346–349) is separated on 1% Tris-borate (TBE) agarose gels, transferred to nylon membranes (Amersham) and probed with ^{32}P-labeled random primed DNA sequences corresponding to a 0.7 kb BamHI/HindIII DNA fragment from pC8 containing a portion of the *rps7/12* plastid targeting sequence. Homoplasmic shoots are rooted aseptically on spectinomycin-containing MS/IBA medium (McBride, K. E. et al. (1994) *PNAS* 91, 7301-7305) and transferred to the greenhouse.

III. Breeding and Seed Production

Example 10: Breeding

The plants obtained via tranformation with a nucleic acid sequence of the present invention can be any of a wide variety of plant species, including those of monocots and dicots; however, the plants used in the method of the invention are preferably selected from the list of agronomically important target crops set forth *supra*. The expression of a gene of the present invention in combination with other characteristics important for production and quality can be incorporated into plant lines through breeding. Breeding approaches and techniques are known in the art. See, for example, Welsh J. R., *Fundamentals of Plant Genetics and Breeding,* John Wiley & Sons, NY (1981); *Crop Breeding,* Wood D. R. (Ed.) American Society of Agronomy Madison, Wisconsin (1983); Mayo O., *The Theory of Plant Breeding*, Second Edition, Clarendon Press, Oxford (1987); Singh, D.P., *Breeding for Resistance to Diseases and Insect Pests*, Springer-Verlag, NY (1986); and Wricke and Weber, *Quantitative Genetics and Selection Plant Breeding*, Walter de Gruyter and Co., Berlin (1986).

The genetic properties engineered into the transgenic seeds and plants described above are passed on by sexual reproduction or vegetative growth and can thus be maintained and propagated in progeny plants. Generally said maintenance and propagation make use of known agricultural methods developed to fit specific purposes such as tilling, sowing or harvesting. Specialized processes such as hydroponics or greenhouse technologies can also be applied. As the growing crop is vulnerable to attack and damages caused by insects or infections as well as to competition by weed plants, measures are undertaken to control weeds, plant diseases, insects, nematodes, and other adverse conditions to improve yield. These include mechanical measures such a tillage of the soil or removal of weeds and infected plants, as well as the application of agrochemicals such as herbicides, fungicides, gametocides, nematicides, growth regulants, ripening agents and insecticides.

Use of the advantageous genetic properties of the transgenic plants and seeds according to the invention can further be made in plant breeding, which aims at the development of plants with improved properties such as tolerance of pests, herbicides, or stress, improved nutritional value, increased yield, or improved structure causing less loss from lodging or shattering. The various breeding steps are characterized by well-defined human intervention such as selecting the lines to be crossed, directing pollination of the parental lines, or selecting appropriate progeny plants. Depending on the desired properties, different breeding measures are taken. The relevant techniques are well known in the art and include but are not limited to hybridization, inbreeding, backcross breeding, multiline breeding, variety blend, interspecific hybridization, aneuploid techniques, etc. Hybridization techniques also include the sterilization of plants to yield male or female sterile plants by mechanical, chemical, or biochemical means. Cross pollination of a male sterile plant with pollen of a different line assures that the genome of the male sterile but female fertile plant will uniformly obtain properties of both parental lines. Thus, the transgenic seeds and plants according to the invention can be used for the breeding of improved plant lines, that for example, increase the effectiveness of conventional methods such as herbicide or pesticide treatment or allow one to dispense with said methods due to their modified genetic properties. Alternatively new crops with improved stress tolerance can be obtained, which, due to their optimized genetic "equipment", yield harvested product of better quality than products that were not able to tolerate comparable adverse developmental conditions.

Example 11: Seed Production

In seed production, germination quality and uniformity of seeds are essential product characteristics. As it is difficult to keep a crop free from other crop and weed seeds, to control seedborne diseases, and to produce seed with good germination, fairly extensive and well-defined seed production practices have been developed by seed producers, who are experienced in the art of growing, conditioning and marketing of pure seed. Thus, it is common practice for the farmer to buy certified seed meeting specific quality standards instead of using seed harvested from his own crop. Propagation material to be used as seeds is customarily treated with a protectant coating comprising herbicides, insecticides, fungicides, bactericides, nematicides, molluscicides, or mixtures thereof. Customarily used protectant coatings comprise compounds such as captan, carboxin, thiram (TMTD®), methalaxyl (Apron®), and pirimiphos-methyl (Actellic®). If desired, these compounds are formulated together with further carriers, surfactants or application-promoting adjuvants customarily employed in the art of formulation to provide protection against damage caused by bacterial, fungal or animal pests. The protectant coatings may be applied by impregnating propagation material with a liquid formulation or by coating with a combined wet or dry formulation. Other methods of application are also possible such as treatment directed at the buds or the fruit.

IV. Disease Resistance Evaluation

Disease resistance evaluation is performed by methods known in the art. *See*, Uknes *et al.* (1993); Görlach *et al.* (1996); Alexander *et al.* (1993). For example, several representative disease resistance assays are described below.

Example 12: *Phytophthora parasitica* (Black Shank) Resistance Assay

Assays for resistance to *Phytophthora parasitica*, the causative organism of black shank, are performed on six-week-old plants grown as described in Alexander *et al.* (1993). Plants are watered, allowed to drain well, and then inoculated by applying 10 ml of a

sporangium suspension (300 sporangia/ml) to the soil. Inoculated plants are kept in a greenhouse maintained at 23-25°C day temperature, and 20-22°C night temperature. The wilt index used for the assay is as follows: 0=no symptoms; 1=no symptoms; 1=some sign of wilting, with reduced turgidity; 2=clear wilting symptoms, but no rotting or stunting; 3=clear wilting symptoms with stunting, but no apparent stem rot; 4=severe wilting, with visible stem rot and some damage to root system; 5=as for 4, but plants near death or dead, and with severe reduction of root system. All assays are scored blind on plants arrayed in a random design.

Example 13: *Pseudomonas syringae* Resistance Assay

Pseudomonas syringae pv. tabaci strain #551 is injected into the two lower leaves of several 6-7-week-old plants at a concentration of 10^6 or 3 x 10^6 per ml in H_20. Six individual plants are evaluated at each time point. *Pseudomonas tabaci* infected plants are rated on a 5 point disease severity scale, 5=100% dead tissue, 0=no symptoms. A T-test (LSD) is conducted on the evaluations for each day and the groupings are indicated after the Mean disease rating value. Values followed by the same letter on that day of evaluation are not statistically significantly different.

Example 14: *Cercospora nicotianae* Resistance Assay

A spore suspension of *Cercospora nicotianae* (ATCC #18366) (100,000-150,000 spores per ml) is sprayed to imminent run-off onto the surface of the leaves. The plants are maintained in 100% humidity for five days. Thereafter the plants are misted with water 5-10 times per day. Six individual plants are evaluated at each time point. *Cercospora nicotianae* is rated on a % leaf area showing disease symptoms basis. A T-test (LSD) is conducted on the evaluations for each day and the groupings are indicated after the Mean disease rating value. Values followed by the same letter on that day of evaluation are not statistically significantly different.

Example 15: *Peronospora parasitica* Resistance Assay

Assays for resistance to *Peronospora parasitica* are performed on plants as described in Uknes *et al*, (1993). Plants are inoculated with a compatible isolate of *P. parasitica* by spraying with a conidial suspension (approximately 5 x 10^4 spores per milliliter). Inoculated plants are incubated under humid conditions at 17° C in a growth chamber with a 14-hr day/10-hr night cycle. Plants are examined at 3-14 days, preferably 7-12 days, after inoculation for the presence of conidiophores. In addition, several plants from each treatment are randomly selected and stained with lactophenol-trypan blue (Keogh *et al.*, 1980) for microscopic examination.

Example 16: Construction of Binary Promoter::Reporter Plasmids

A binary promoter::reporter plasmid (pNOV2374) for Arabidopsis transformation was constructed. The regulatory/promoter sequence was fused to the GUS reporter gene (Jefferson et al. (1987) EMBO J 6: 3901-3907) by recombination using GATEWAY™ Technology according to the manufacturer's protocol as described in the Instruction Manual (LifeTechnologies, Rockville, MD). The construct was then transformed into *Agrobacterium tumefaciens* strains by electroporation.

pNOV2374 is a binary vector with a VS1 origin of replication, a copy of the *Agrobacterium* virG gene in the backbone, and a Basta® resistance selectable marker cassette between the left and right border sequences of the T-DNA. The Basta® selectable marker cassette comprises the *Agrobacterium tumefaciens* manopine synthase promoter (AtMas) (Barker et al., Plant Mol. Biol. 2, 335-350 (1983)) operably linked to the gene encoding Basta® resistance (i.e., the "BAR" gene, phosphinothricin acetyl transferase) (White et al., Nucl Acids Res 18: 1062 (1990)) and the 35S terminator. The AtMas promoter, the BAR coding sequence, and the 35S terminator are located at nt 4211 to nt 4679, nt 4680 to nt 5228, and nt 5263 to nt 5488, respectively, of pNOV2374. The vector contains GATEWAY™ recombination components, which were introduced into the binary vector backbone by ligating a blunt-ended cassette containing attR sites, ccdB, and a cholramphenicol resistance marker using the GATEWAY™ Vector Conversion System (LifeTechnologies, Rockville,

MD). The GATEWAY™ cassette was located between nt 126 and 1818 of pNOV2374. The promoter cassettes were inserted through an LR recombination reaction whereby the DNA sequence of pNOV2374 between nt 126 and nt 1818 were removed and replaced with the promoter of interest flanked by att sequences. The recombination resulted in the promoter sequence being fused to the GUS reporter gene with an intron sequence (GIG). The GIG gene (SEQ ID NO:29) contains the ST-LS1 intron from *Solanum tuberosum* at nt 385 to nt 576 of GUS (obtained from Dr. Stanton Gelvin, Purdue University, and described in Narasimhulu, et al. (1996) Plant Cell 8: 873-886.). The orientation of the selectable marker and promoter-reporter cassettes in the binary vector construct was as follows:

RB -- NI16 promoter fragment (i.e., SEQ ID NO:24, SEQ ID NO:27, or SEQ ID NO:28) + GIG gene (SEQ ID NO:29) + nos -- AtMas + BAR + 35S term -- LB

For the purpose of comparing promoter activity, additional constructs were produced with the known Arabidopsis PR1 (SEQ ID NO:30) and SMAS (SEQ ID NO:31) promoters (*see* Plant J. 16 (2) 223-233 (1998) and Plant Mol. Biol. 20 (2) 219-233 (1992), respectively) operatively linked to the GIG gene (SEQ ID NO:29) and the nos promoter. The orientation of the selectable marker and promoter-reporter cassette in these binary vector constructs is as follows:

RB -- AtPR1 promoter (SEQ ID NO:30) + GIG gene (SEQ ID NO:29) + nos -- AtMas + BAR + 35S term -- LB

RB -- SMAS promoter (SEQ ID NO:31) + GIG gene (SEQ ID NO:29) + nos -- AtMas + BAR + 35S term -- LB

Example 17: Arabidopsis Transformation

1. Plant Preparation and Growth

Arabidopsis seeds were sown on moistened Fafard Germinating Mix at a density of 9 seeds per 4" square pot, placed in a flat, covered with a plastic dome to retain moisture, and then moved to a growth chamber. Following germination, the dome was removed and plants were grown for 3-5 weeks under short day (8 hrs light) conditions to encourage vegetative growth and the production of large plants with many flowers. Flowering was induced by

providing long day (16 hrs. light) conditions for 2-3 weeks, at which time the plants were ready for dip inoculation into *Agrobacterium* to generate transgenic plants.

2. Agrobacterium Transformation, Culture Growth, and Preparation for Plant Infiltration

The binary promoter::reporter plasmids were introduced into Agrobacteria by electroporation. The binary plasmid confers spectinomycin resistance to the bacteria, allowing cells containing the plasmid to be selected by the growth of colonies on plates of LB + spectinomycin (50 mg/L). The presence of the correct promoter::GUS plasmid was confirmed by sequence analysis of the plasmid DNA isolated from the bacteria.

Two days prior to plant transformation, 5 mL cultures of LB + spectinomycin (50 mg/L) were inoculated with the Agrobacterium strain containing the binary promoter::GUS plasmid and incubated at 30° C for ~24 hours. Each 5 mL culture was then transferred to 500 mL of LB + spectinomycin (50mg/L) and incubated for ~24 hours at 30° C. Each 500 mL culture was transferred to a centrifuge bottle and centrifuged at 5000 rpm for 10 minutes in a Sorvall Centrifuge. The supernatant was removed and the pelleted Agrobacterium cells were retained. The Agrobacterium cells were resuspended in 500 mL of modified Infiltration Media (IM+MOD: 50g/L sucrose, 10mM MgCl, 10 uM benzylaminopurine) to which 50 ul of Silwet L-77 (Dupont) had been added.

3. Plant Transformation by Dip Infiltration

Resuspended cells were poured into 1L tri-pour beakers. Flowering plants were inverted into the culture, making sure all inflorescences were covered with the bacteria. The beakers were gently agitated for 30 seconds, keeping all inflorescence tissue submerged. Plants were returned to the growth chamber following dip inoculation of the Agrobacterium. A second dip may be performed 5 days later to increase transformation frequency. Seeds were harvested ~4 to 6 weeks after transformation.

4. Selection of Transgenic Arabidopsis

Seeds from transformed Arabidopsis plants were sown on moistened Fafard Germinating Mix in a flat, covered with a dome to retain moisture, and then placed in a growth chamber. Following germination, seedlings were sprayed with the herbicide BASTA®. Transgenic plants are BASTA® resistant due to the presence of the BAR gene in the binary promoter::GUS plasmid.

Example 18: Promoter Activity Induction Assays

1. Treatment with Salicylic Acid

Three to four week old plants were sprayed with 5mM salicylic acid (SA) dissolved in H_20. The treatment was applied as a fine mist to the leaves. Leaf tissue was harvested from plants for the ß-glucuronidase (GUS) florescence assay prior to SA application (T_0) and at various time intervals after application.

2. Treatment by Buffer Injection or Pathogen Inoculation

Leaves of three to four week old plants were injected with 10mM $MgCl_2$ (buffer) or inoculated with the pathogen *Pseudomonas syringe maculicula* containing the avrRPM1 avirulence gene, which elicits a hypersensitive response (HR) in the Ws cultivar of Arabidopsis. Leaf tissue was harvested from plants immediately after treatment with the buffer or pathogen (T_0) and at various time intervals after injection. Promoter activity was evaluated by ß-glucuronidase (GUS) florescence.

Example 19: Reporter Gene Assays

Promoter activity was evaluated qualitatively and quantitatively using histochemical and florescence assays for expression of the ß-glucuronidase (GUS) enzyme.

1. Histochemical ß-Glucuronidase (GUS) Assay

For qualitative evaluation of promoter activity, various Arabidopsis tissues and organs were used in GUS histochemical assays. Either whole organs or pieces of tissue were dipped into GUS staining solution. The GUS staining solution contained 1 mM 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc, Duchefa, 20 mM stock in DMSO), 100 mM Na-phosphate buffer pH 7.0, 10 mM EDTA pH 8.0, and 0.1% Triton X100. Tissue samples were incubated at 37° C for 1-16 hours. If needed, samples can be cleared with several washes of 70% EtOH to remove chlorophyll. Following staining, tissues were viewed under a light microscope to evaluate the blue staining showing the GUS expression pattern.

2. ß-Glucuronidase (GUS) Florescence Assay

For quantitative analysis of promoter activity following induction treatments or in various Arabidopsis tissues and organs, GUS expression is measured florometrically. Tissue samples were harvested and ground in ice cold GUS extraction buffer (50mM Na2HPO4 pH 7.0, 5mM DTT, 1mM Na2EDTA, 0.1% TritonX100, 0.1% sarcosyl). Ground samples were spun in a microfuge at 10,000 rpm for 15 minutes at 4° C. Following centrifugation, the supernatant was removed for the GUS assay and for a protein concentration determination.

To measure GUS activity, the plant extract was assayed in GUS assay buffer (50mM Na2HPO4 pH 7.0, 5mM DTT, 1mM Na2EDTA, 0.1% TritonX100, 0.1% sarcosyl, 1mM 4-Methylumbelliferyl-beta-D-glucuronic acid dihydrate (MUG)), pre-warmed to 37° C. Reactions were incubated, and 100 uL aliquots were removed at 10 minute intervals over 30 minutes to stop the reaction by adding the 100 uL aliquots to tubes containing 900 uL of 2% Na_2CO_3. The stopped reactions were then read on a Tecan Spectroflourometer at 365nm excitation and 455 emission wavelengths. Protein concentrations were determined using the BCA assay following the manufacturer's protocol. GUS activity is expressed as pmol MU/mg protein/min.

Table 4

Comparison of NI16 promoter fragments' activity following salicylic acid (SA) Induction by SA application

Induction of NI16 Promoter Activity by SA					
GUS Activity (pmol/mg protein/min) Induced by SA					
Fragment Length	T_0	2h	4h	8h	24h
274	19.4	27.5	58.7	150.8	230.3
544	12.1	52.2	72.5	194.9	205.4
962	39.7	86.8	260.8	467.7	1457.5
PR1	16.8	57.7	254.7	206.5	393.1
Fold-change relative to T_0 (uninduced)					
Fragment Length	T_0	2h	4h	8h	24h
274	1.0	1.4	3.0	7.8	11.9
544	1.0	4.3	6.0	16.2	17.0
962	1.0	2.2	6.6	11.8	36.7
PR1	1.0	3.4	15.2	12.3	23.4

Table 5

NI16 962 bp promoter fragment activity following pathogen treatment Induction by Buffer or Pathogen-inoculation

Induction of NI16 Promoter Activity following Buffer or Pathogen Inoculation					
GUS Activity (pmol/mg protein/min)					
		0h	4h	8h	24h
Ws	Buffer	1.4	4.7	4.0	7.8
	Pathogen	2.5	6.5	4.1	8.1
SMAS	Buffer	659.3	1055.1	952.6	2008.0
	Pathogen	809.2	1176.6	947.3	2166.3
PR1	Buffer	5.3	40.2	18.1	65.9
	Pathogen	8.7	10.1	53.1	224.9
NI16	Buffer	63.2	91.3	113.7	271.4
(962)	Pathogen	26.8	104.4	173.8	525.8
Fold-change relative to Time 0 (untreated)					
		0h	4h	8h	24h
Ws	Buffer	1.00	3.43	2.93	5.72
	Pathogen	1.00	2.60	1.64	3.25
SMAS	Buffer	1.00	1.60	1.44	3.05
	Pathogen	1.00	1.45	1.17	2.68
PR1	Buffer	1.00	7.52	3.38	12.33
	Pathogen	1.00	1.16	6.12	25.97
NI16	Buffer	1.00	3.07	3.43	8.25
(962)	Pathogen	1.00	3.57	7.01	24.53

All publications, published patent documents, and patent applications cited in this specification are indicative of the level of skill in the art(s) to which the invention pertains. All publications, published patent documents, and patent applications cited herein are hereby incorporated by reference to the same extent as though each individual publication, published patent document, or patent application was specifically and individually indicated as being incorporated by reference.

The foregoing describes the invention with reference to various embodiments and examples. No particular embodiment, example, or element of a particular embodiment or example is to be construed as a critical, required, or essential element or feature of any or all of the claims. As used herein, the terms "comprises," "comprising," "includes," "including," "contains," "containing," and any variations thereof, are intended to cover a non-exclusive inclusion, such that a process, method, product-by-process, or composition of matter that comprises, includes, or contains an element or list of elements does not include only those elements but may include other elements not expressly listed or inherent to such process, method, product-by-process, or composition of matter. Further, no element described herein is required for the practice of the invention unless expressly described as "essential" or "critical."

It will be appreciated that various modifications and substitutions can be made to the disclosed embodiments without departing from the scope of the invention as set forth in the claims below. The specification, including the drawings and examples, is to be regarded in an illustrative manner, rather than a restrictive one, and all such modifications and substitutions are intended to be included within the scope of the invention. Accordingly, the scope of the invention should be determined by the appended claims and their legal equivalents, rather then by the examples given above. For example, the steps recited in any of the method or process claims may be executed in any feasible order and are not limited to an order presented in any of claims.

REFERENCES

The references cited herein are indicative of the current state of the art. Each of the following is incorporated by reference into the instant disclosure.

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